UNIVERSITY of TASMANIA

Inhibition of Platelet Aggregation by Vanilloid-like Agents: Investigation of Possible Mechanisms

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Declarations

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Statement of Co-Authorship of Jointly Published Work

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ADP	Adenosine diphosphate	MP	Microparticles
ATP	Adenosine triphosphate	NADA	N-arachidonoyl-dopamine
AEA	Anandamide	OLDA	N-oleoyldopamine
APS	Antiphospholipid syndrome	OCS	Open canalicular system
AA	Arachidonic acid	PAR	Protease-activated receptor
2-AG	2-arachidonoylglycerol	PF4	Platelet factor 4
CGRP	Calcitonin gene-related peptide	PLA	Phospholipase A
CB	Cannabinoid receptors	PLC	Phospholipase C
CAP	Capsaicin	PIP_2	Phosphatidylinositol-4,5-bisphosphate
CVD	Cardiovascular disease	PMPs	Platelet-derived Microparticles
CNS	Central nervous system	PGH_2	Prostaglandin H_2
COX-1	Cyclooxygenase-1	PKC	Protein kinase C
DC	Dendritic cell	PRI	Platelet reactivity index
DHC	Dihydrocapsaicin	RTX	Resiniferatoxin
FAAH	Fatty acid amide hydrolase	SLE	Systemic lupus erythrematosus
fL	Femtolitres	THC	Delta-9-tetrahydrocannabinol
GPCRs	G protein-coupled receptors	TXA2	Thromboxane A2
IP_3	1,4,5- inositol triphosphate	TXB2	Thromboxane B2
IFN-α	Interferon-α	TRPV1	Transient receptor potential vanilloid 1
JAMs	Junctional adhesion molecules	VASP	Vasodilator-stimulated phosphoprotein
LDL	Low-density lipoprotein	vWF	Von Willebrand factor

General Abstract

Platelets are non-nucleated cell that play a central role in maintaining the haemostatic process. They also contribute to thrombotic events, and the initiation and progression of atherosclerosis. Antiplatelet medications such as aspirin have been shown to have a beneficial effect in the primary and secondary prevention of cardiovascular diseases. However, their use can have significant side effects, including gastrointestinal ulceration, gastritis and bleeding.

It has been shown that vanilloid-like agents, including plant-derived vanilloids (capsaicin (CAP) and dihydrocapsaicin (DHC)), and endogenous vanilloids (N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA)), individually inhibit *in vitro* aggregation in platelets obtained from healthy donors. Thus, the overall aim of this thesis was to investigate the possible mechanism(s) by which vanilloids inhibit platelet aggregation, both individually and in combination. This is important especially since some studies have shown that CAP has a gastro-protective effect against mucosal damage induced by aspirin, indomethacin and ethanol. Moreover, regular chilli consumption has been reported to decrease the incidence of peptic ulcers, control glucose and insulin levels, making chilli a potential nutraceutical.

This thesis consists of six chapters. Chapter 1 is a review and critique of the literature on platelet structure and function, transient receptor potential vanilloid-1 (TRPV1) channels and cannabinoid (CB) receptors in health and disease, vanilloids and their clinical applications, the manifestations of systemic lupus erythematosus (SLE), and the role platelets in SLE. Chapters two through five present the related background,

research experiments and data generated that detail the mechanistic investigations of the inhibitory effects of vanilloids on platelet function. The last Chapter 6 is a comprehensive discussion of all the investigations conducted during candidature, and includes directions for future work stemming from this research.

Vanilloid-like agents mediate their actions on neurons and other cells through TRPV1 channels and/or CB receptors. The aims of the investigations described in Chapter 2 were to firstly to confirm TRPV1 expression on human platelets, and then to determine whether vanilloid inhibition of *in vitro* platelet aggregation was receptor mediated, i.e., through TRPV1 channels, and/or CB1 or CB2 receptors. Platelets were obtained from healthy volunteers for all experiments unless otherwise stated. Expression of TRPV1 in platelets was confirmed, although to my knowledge, this is the first time this has been demonstrated using confocal microscopy. Furthermore, the inhibitory effects of vanilloids on *in vitro* platelet aggregation induced by collagen, Adenosine diphosphate (ADP) and arachidonic acid (AA) were found not to be TRPV1-, CB1- or CB2- receptor mediated. However, blocking TRPV1 and CB2 receptors appear to enhance OLDA and CAP inhibitory action on platelets.

After excluding a definite role for TRPV1, CB1 and CB2 receptors in the action of vanilloid on platelets, other possible mechanisms were investigated in Chapter 3. My previous Masters work showed that the endovanilloids, OLDA and NADA, as well as the high concentrations (100-25 μM) of plant-derived vanilloid, CAP, significantly inhibit *in vitro* ADP-induced platelet aggregation. Furthermore, CAP, DHC and NADA inhibited AA-induced aggregation, whereas NADA and OLDA only inhibited aggregation induced by a low concentration of collagen (4 μg/mL, but

not 8 μg/mL). Thus, the focus of this thesis was whether vanilloids exert their action on platelets by interfering with 1) ADP receptors by measuring vasodilator-stimulated phosphoprotein (VASP) phosphorylation level, dense- (5-hydroxytryptamine (5-HT)) release, and/or α-granules (platelet factor 4 (PF4) and β-thromboglobulin (β-TG)) release, and/or 2) the AA metabolic pathway in platelets. Furthermore, the effect of vanilloids on platelet-derived microparticles (PMP) was also determined.

NADA significantly increased VASP phosphorylation (17% ± 2.2, $p<0.05$) compared to control (no treatment control), indicate a potential involvement of ADP receptor. In addition, OLDA also increased VASP phosphorylation by 13.4%± 2.7, $p=0.12$ but the result was not statistically significant. However, none of the vanilloids tested produced a significant effect on PF4, β-TG or 5-HT release from ADP-activated platelets. Under AA stimulation, thromboxane B2 (TXB2) formation decreased significantly in the presence of 50 μM CAP (10.7%, $p<0.001$), whereas in the presence of 50 μM DHC, the decrease in TXB2 was not significant (4.6%, $p=0.8$). In contrast, OLDA and NADA had no effect on TXB2 formation compared to AA alone. In ADP- and AA-stimulated platelets, vanilloids had no effect on PMP release. Taken together, these results suggest that NADA and possibly OLDA inhibits *in vitro* platelet aggregation through interference with the ADP receptor, P_2Y_{12}, as VASP phosphorylation increased significantly in its presence. Moreover, CAP and perhaps DHC, inhibit the AA pathway, as TXB2 formation was significantly decreased. Finally, no changes in circulating PMP in the presence of CAP, DHC, OLDA and NADA were observed, suggesting that they do not affect the pathway that leads to PMP formation. Although the precise mechanism(s) that

produce PMP is(are) not well understood, alterations in phospholipid symmetry and cytoskeleton rearrangement appear to be essential.

Pepper fruits (chilli) are the main source of vanilloids, CAP and DHC, which are usually present in 60:40 ratio. In Chapter 4, the effects of CAP and DHC both individually, and in combination (CAP:DHC, 60:40), on AA-, ADP-, and collagen-induced *in vitro* platelet aggregation were investigated and compared. Additionally, their effects on platelet count and TXB2 formation were determined to assess the combination toxicity toward platelets and their mechanism of action, respectively. Under AA stimulation, 12.5 μM CAP and DHC inhibited aggregation by 23.2% and 25.3%, respectively compared to control (both $p<0.01$). Interestingly, combination of CAP and DHC (7.5:5 μM) produced further inhibition 57.5%, $p<0.001$, compared to control (no treatment control). However, CAP and DHC individually, and in combination, had no effect on ADP- or collagen-induced platelet aggregation. Incubation of platelets with vanilloids, individually or in combination, did not significantly affect the platelet count. The 60:40 CAP:DHC (7.5:5 μM) combination significantly inhibited ($p<0.001$) TXB2 formation compared to the individual vanilloids. These results indicate that CAP and DHC in combination act synergistically to inhibit the AA metabolic pathway. Therefore, the combination of chilli pepper-derived vanilloids exhibits a stronger antiplatelet effect than the individual vanilloids, which opens up a new opportunity for research.

Finally, a pilot study was conducted that investigated the effect of vanilloid-like agents, CAP, DHC, OLDA and NADA (0-50 μM) on ADP-(5 μM) and collagen-(4 μg/mL) induced aggregation of platelets obtained from SLE patients (Chapter 5). As

all patients were on non-steroidal anti-inflammatory drugs, AA-induced aggregation has been excluded. These patients have a higher risk of thrombotic events and the development of atherosclerosis compared to the general population that may be associated with enhanced platelet activation. CAP, DHC, OLDA and NADA, in contrast to their inhibitory effects on platelets from healthy individuals, had no effect on ADP-induced aggregation of platelets from SLE patients. Similarly, CAP, DHC and OLDA did not influence aggregation induced by collagen. However, NADA inhibited collagen-induced aggregation in a concentration-dependent manner (0 vs 50 μM; %AUC, 44.8±6.5 vs 34.7±7.8, $p<0.001$) and (%MAX, 60.8±7.7% vs 37.8±9.7%, $p<0.001$ n = 5). These results suggest that the pathway(s) through which CAP, DHC and OLDA inhibit platelet aggregation in healthy platelets may be impaired in SLE, and/or affected by the medications used to treat the manifestations of SLE.

In summary, through these studies I have generated new data and knowledge on the mechanism of action of vanilloid-like agents on platelets and platelet aggregation. The major outcomes are as follows. First, TRPV1 expression on platelets has been confirmed using confocal microscopy. Second, inhibition of *in vitro* platelet aggregation by vanilloid-like agents appears to be independent of a direct interaction with TRPV1 channels or CB receptors. Third, CAP and DHC appear to inhibit *in vitro* platelet aggregation by interfering with the AA-pathway, whereas NADA and OLDA do so by interfering with and/or blocking the ADP receptor, P1Y12. Fourth, CAP, DHC, NADA and OLDA have no effect on the release of PMP from ADP- and AA- stimulated platelets. Fifth, low concentrations of a CAP and DHC combination (60:40) have a greater inhibitory effect on *in vitro* platelet aggregation through the

AA-pathway, compared to a higher concentration of the individual vanilloid. Finally, unlike findings in platelets from healthy individuals, only NADA appears to have an inhibitory effect on platelets from patients with SLE. The data presented in this thesis can be used as a basis for the design of future studies that may investigate the effect of vanilloid-like agents using *in vivo* models as well as on other patients with high risk of thrombosis and atherosclerosis to test the viability of chilli pepper as nutraceutical.

1. Chapter 1: Literature Review

1.1. Introduction

Platelets are essential for primary haemostasis and endothelium repair, however they also play a key role in chronic inflammation and atherosclerosis, the major cause of cardiovascular disease (CVD) (Zucker, 1980). Platelets participate in chronic inflammation by forming and extending atherosclerotic plaques (Lusis, 2000, Ross, 1999). Therapeutic agents that suppress platelet aggregation are therefore beneficial in the prevention and treatment of CVD.

The active principles of hot chilli peppers, capsaicin (CAP) and dihydrocapsaicin (DHC), as well as the endovanilloids, N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA) have been reported to inhibit *in vitro* platelet aggregation (Almaghrabi et al., 2014, Adams et al., 2009, Raghavendra and Naidu, 2009), however, their precise mechanism(s) of action are yet to be determined.

CAP, NADA and OLDA appear to exert their action through transient receptor potential vanilloid 1 (TRPV1)-dependent and -independent mechanisms in neuronal tissue (Caterina and Julius, 2001). TRPV1 is a non-selective cation channel that is stimulated by a wide variety of agents and physical factors, including plant-derived vanilloids (e.g. CAP), endovanilloids (e.g. OLDA and NADA), heat (> 43 ^{0}C) and acid (pH < 7) (Caterina and Julius, 2001, Zhong and Wang, 2008). Endovanilloids also have an affinity for cannabinoid (CB) receptors, CB1 and CB2, and are hence also termed endocannabinoids (Howlett et al., 2002). TRPV1 channels, as well as CB1 and CB2 receptors, are expressed in platelets, although the precise role(s) of these receptors in platelet function is unclear (Harper et al., 2009, Deusch et al.,

2004, Catani et al., 2010).

CVD and atherosclerosis are well-described clinical manifestations in autoimmune diseases such as systemic lupus erythematosus (SLE) (Ward, 1999, Gladman and Urowitz, 1987). The worldwide prevalence of SLE varies from 4.3 to 150 per 100 000 population (Chakravarty et al., 2007, Pons-Estel et al., 2010, Jakes et al., 2012), with high prevalence in women in childbearing age and certain ethnic groups (Askanase et al., 2012, Maidhof and Hilas, 2012). SLE is a prototypic autoimmune disease characterised by a perturbed immune response against self, with the consequent production of autoantibodies. Some of these autoantibodies form immune complexes that are responsible for tissue inflammation that directly contribute to the development of several clinical manifestations of the disease (Ruiz-Irastorza et al., 2001). In SLE, platelets are abnormally activated, which leads to the release of inflammatory mediators into the local environment. As a result, the adhesive and chemotactic properties of endothelial cells change and increase the risk of atherosclerosis, despite thrombocytopenia in some cases (Gawaz et al., 2005, West and Johnson, 1988). Understanding the mechanism(s) of action of vanilloids and endovanilloids compounds in platelets and how they might affect platelet activation in SLE patients is important, as these compounds may be developed as novel anti-platelet nutraceutical agents.

1.2. Platelets

Platelets are anuclear, small, irregularly shaped fragments of megakaryocyte cytoplasm (Avraham, 1993). Their main role is to maintain normal haemostasis and repair wounds (Hoak, 1988, Jurk and Kehrel, 2005), but they also contribute to other

pathophysiological events such as atherosclerosis, arterial thrombosis and acute thrombotic events.

1.2.1. Structure and Function

Platelets are discoid in shape, with approximate dimensions of 2.0-9.0 by 0.5 μm, and an average volume of 7-11 femtolitres (fL). They typically circulate in the blood for approximately ten days at a concentration of 150 - 450 x 10^9/L (George, 2000). Moreover, they have multiple roles and contribute to many physiological and pathological processes such as haemostasis, thrombosis, wound repair, vessel constriction and clot retraction, tumour growth, host defense and inflammation (Ruggeri, 2002, Harrison, 2005).

Although platelets are cell fragments, they contain many structures that are crucial for their function. These include mitochondria, which regulate the pro-thrombotic actions function of platelets and the initiation of apoptosis (Jobe et al., 2008, Vanags et al., 1997). An open canalicular system (OCS) serves as a two-way passageway for exchange of substances between granular contents of the platelet and the blood plasma, and as a reservoir of plasma membrane, membrane proteins, and receptors (Escolar and White, 1991, White, 1972). The dense tubular system plays an integral role in regulating platelet activation, as a main calcium releasing and sequestering organelle (White, 1972, Brass, 1984, Gerrard et al., 1976). The plasma membrane cytoskeleton forms 30 percent of the total platelet proteins, which include actin and tubulin. The main function of the cytoskeleton is the maintenance of the discoid shape of resting platelets and rapid changes in shape in activated platelets. Moreover, the platelet cytoskeleton is responsible for cellular movements, such as transport of organelles across the platelet chromosome segregation during mitosis (Fox, 1993,

Olorundare et al., 1993, Fox, 2001). Furthermore, platelets have secretory granules, alpha (α) granules, dense granules, lysosomes and peroxisomes, which contain proteins that regulate angiogenesis, stimulate platelet adhesion, enhance vascular repair and promote cell-cell interaction (Figure 1-1) (Kamath et al., 2001, Fukami and Salganicoff, 1977, Stenberg et al., 1984, Siess, 1989). Furthermore, platelets express different receptors that modulate their function (De Botton et al., 2002). Platelets react to endothelial damage via tightly controlled functional responses, including adhesion, granule release, activation and aggregation, exposure of procoagulant surface, microparticle formation and clot retraction. As a result of these responses, a haemostatic plug forms to occlude the damage and prevent bleeding (Rodgers, 1999, Harrison, 2005).

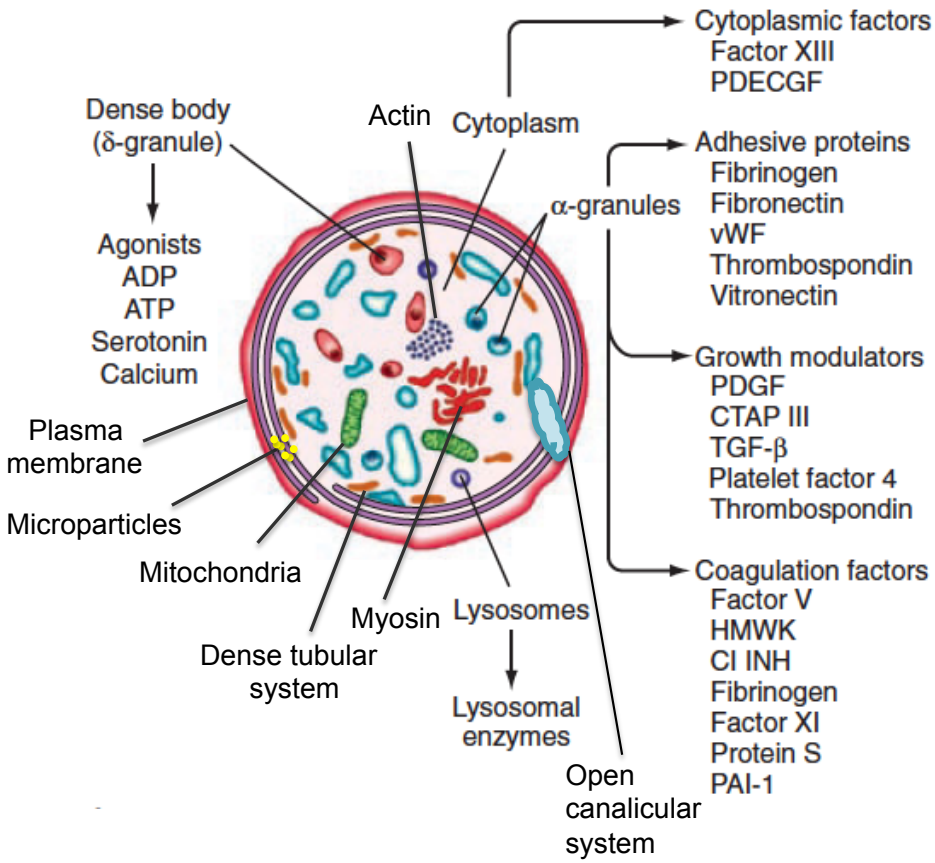

Figure 1-1 Platelets structure and contents of secretory granules

Substances released by platelets and their intra-platelet sources. Illustrated are some of the bioactive substances released from dense bodies, α-granules, lysosomes, cytoplasm, and platelet membrane. C1 INH, C1 inhibitor; CTAP, connective tissue-activating peptide; HMWK, high-molecular-weight kininogen; PAI-1, plasminogen activator inhibitor 1; PDECGF, platelet-derived endothelial cell growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; and vWF, von Willebrand factor. The figure was sourced and modified from Hoffman et al. (2008).

1.2.1.1. Activation and Aggregation

Platelet activation is divided into three overlapping stages: initiation, extension and perpetuation (Hoffman et al., 2008). The damaged endothelium exposes collagen, which attracts and then activates platelets via attachment to von Willebrand factor (vWF) multimers. A platelet monolayer forms over the exposed collagen that stimulates thrombin generation and subsequent platelet aggregation. Thrombin and collagen normally initiate *in vivo* platelet activation, however several agonists can stimulate *in vitro* platelet aggregation (Falati et al., 2003, Del Conde et al., 2005).

The platelet surface receptors, glycoprotein Ib/V/IX (GPIb-V-IX), integrin $\alpha_{IIb}\beta_3$ (followed by intracellular signaling through $\alpha_2\beta_1$) and GPVI, assist vWF-dependent binding of platelets. As a result, more platelets are attached to the damaged endothelium and create a surface for further platelet-platelet interaction (Massberg et al., 2003). Moreover, collagen receptors help to capture fast-moving platelets, which lead to platelet activation and cytoskeleton reorganisation. vWF propagates this process by increasing the affinity of the collagen binding site for platelets and stabilises the adhesion of platelets to exposed endothelium collagen (Massberg et al., 2003, Nieswandt et al., 2001, Nieuwenhuis et al., 1985, Sixma et al., 1997, Kato et al., 2003).

In thrombotic and inflammatory diseases, thrombin initiates platelet activation through proteolytic cleavage and stimulation of a subfamily of G Protein-Coupled Receptors (GPCRs) known as Protease-Activated Receptors (PAR1 and PAR4). The initiation stage of platelet activation is adequate to form a platelet plug, but not to arrest bleeding. The second phase of platelet activation is initiated when more platelets are attracted, activated and aggregated on the collagen-bound monolayer (Hoffman et al., 2008). Consequently, secretion of agonists such as thrombin, ADP and thromboxane A_2 (TXA2) increases intra-platelet calcium, that in turn activates phospholipase C (PLC) and phospholipase A (PLA), recruiting further platelets to the injured endothelium. Phosphatidylinositol-4,5-bisphosphate (PIP_2) is hydrolysed by PLCγ2, an isoform of PLC, to form diacylglycerol and 1,4,5- inositol triphosphate (IP_3). IP_3 opens Ca^{2+} channels in the platelet-dense tubular system, which increases cytosolic Ca^{2+} influx through the platelet plasma membrane. In contrast, PLA hydrolyses arachidonic acid (AA) from phospholipids in the platelet membrane, with cyclooxygenase-1 (COX-1) then converting AA to prostaglandin H_2 (PGH_2).

Thromboxane synthase generates TXA2 from PGH_2, with thromboxane B_2 (TXB2) subsequently produced by a non-enzymatic hydration of TXA2 (Figure 1-2) (Kulkarni et al., 2004, Nesbitt et al., 2003).

Circulating or locally secreted catecholamines cause vasoconstriction and stimulate activation of platelets by propagating the effects of platelet agonists. Most platelet agonists exert their effect of extending the platelet plug through GPCRs, which are the largest family of membrane proteins in human genome (Fredriksson et al., 2003, Pierce et al., 2002, Vassilatis et al., 2003). Chemically different ligand groups, such as nucleotides, lipids, amines, ions, proteases and peptides can stimulate GPCRs (Bockaert et al., 2002). As a result of their ability to interact with an array of functionally diverse heterotrimeric guanine nucleotide-binding proteins (G proteins), activation of GPCRs by agonist(s) can stimulate different signaling pathways to alter cellular functions (Cabrera-Vera et al., 2003, Wettschureck and Offermanns, 2005). The plethoric versatility of G protein-mediated signaling may explain why it is well-suited for its function as a primary mediator of the second phase of platelet activation in the haemostasis and thrombosis processes. These processes require rapid action and coordination of various mediators to activate and recruit more platelets into the developing thrombus (Offermanns, 2006).

During the perpetuation stage, the platelet plug is stabilised to stop premature disaggregation. The aggregated platelets stabilise mainly by the cohesive strength of the binding between $\alpha_{IIb}\beta_3$ and fibrin, fibrinogen or vWF (Shattil and Newman, 2004). In addition, there are other molecules that facilitate adhesion and intracellular signaling, apart from integrins such as junctional adhesion molecules (JAMs) (Muller, 2003, Bazzoni, 2003), signaling lymphocytic activation molecule (SLAM;

CD150) (Krause et al., 2000, Martin et al., 2001, Nanda et al., 2005) and platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31) (Newman and Newman, 2003). Activation of platelets is a very dynamic process where receptors and effector pathways regulate each stage of this process (Hoffman et al., 2008).

1.2.1.2. Platelets Adhesion Receptors

Platelets normally circulate within the blood in an inactive state without interacting with other platelets or cells. Following exposure to stimulatory agonists or vascular injury, platelets become more adhesive. Most adhesive proteins in platelets belong to the integrin group, a widely distributed family of heterodimeric cell surface molecules (having two subunits, α and β), which share some functional, structural and immunochemical characteristics (Hynes, 1992, Fitzgerald et al., 1987). Each α-subunit joins a β-subunit via a non-covalent bond, forming an efficient adhesive protein receptor. The major β-subunits expressed on platelets are β_1 and β_3 (β_2 at low levels), along with five α-subunits (Philippeaux et al., 1996). The $\alpha_{IIb}\beta_3$ integrin (GPIIb/IIIa) is unique to megakaryocytes/platelets and plays an important role in platelet aggregation and tumor cell-platelet interaction (Grossi et al., 1988, Boukerche et al., 1989, Honn et al., 1992).

GPIb-V-IX is a platelet surface molecule that is involved in platelet adhesion as a receptor for vWF, but is not one of the integrin family (Andrews et al., 2003). vWF mediates reversible rapid adhesion of platelets which allows the rolling of platelets along the surface of the disrupted endothelium (Sadler, 2002). This rolling may temporarily bridge the platelet to subendothelial matrix contents until a more stable

adhesive bond is formed. The binding between GPIb-V-IX and vWF leads to intracellular signaling events that result in activation of $\alpha_{IIb}\beta_3$ and platelet aggregation (Berndt et al., 2001, Savage et al., 1992). Furthermore, the role of GPIb-V-IX in haemostasis is not limited to vWF, but also as a binding site for thrombin, which has an important function in the production of PMPs and procoagulant activity (Okumura and Jamieson, 1976, Moroi et al., 1982).

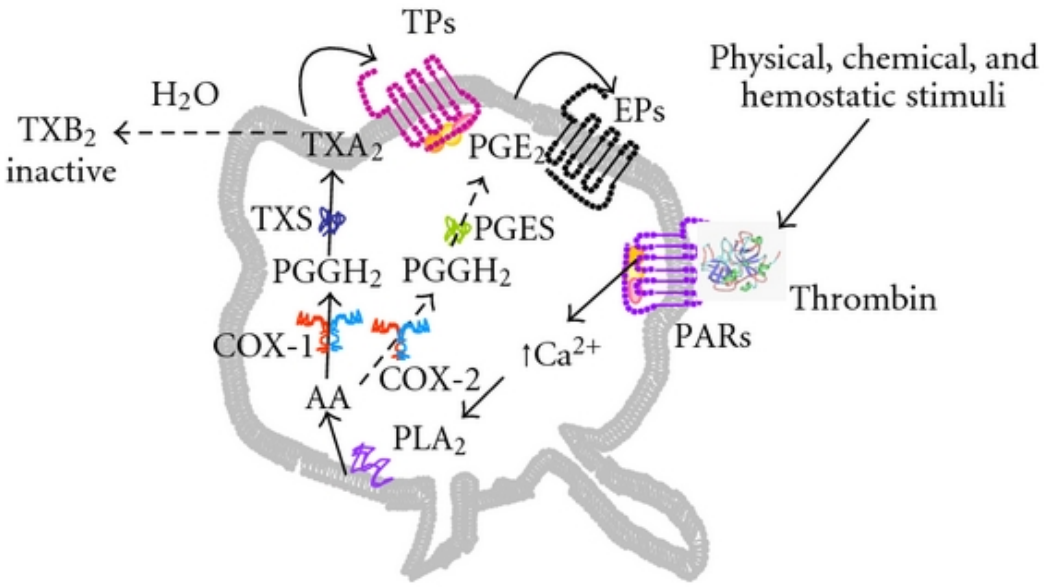

Figure 1-2 Arachidonic acid pathway in platelets

Cyclooxygenase-dependent arachidonic acid pathway in platelets. Thrombin, generated *in vivo* or *ex vivo* by several chemical or physical stimuli, activates its protease-activated receptors (PARs) increasing intraplatelet calcium, which triggers phospholipases (PL) A2-dependent cleavage of arachidonic acid (AA) from plasma membranes. AA is the enzymatic substrate of cyclooxygenase (COX)-1 and -2. COX-1-dependent AA path in platelets generates mainly TXA2, which amplifies platelet activation by binding to its platelet receptors (TPs). COX-2-dependent AA path in normal platelets is less prominent and generates mainly PGE2, which acts as a positive modulator of platelet response to other agonists by binding to its platelet receptors (EPs). TXA2 both *in vivo* or *ex vivo* is nonenzymatically hydrolised to TXB2, which is biologically inactive but stable, and can be measured in *ex vivo* assays or undergoes further hepatic enzymatic biotransformation *in vivo*. Image sourced from Rocca and Petrucci, 2012 without modifications (Rocca and Petrucci, 2012).

Subendothelial collagen is a well-known potent platelet agonist that initiates platelet activation and adhesion. There are four collagen receptors, $\alpha_2\beta_1$ and GPVI bind

directly to collagen, and $\alpha_{IIb}\beta_3$ and GPIb-V-IX bind to collagen through vWF. $\alpha_2\beta_1$ (also known as VLA-2 and platelet GPIa-IIa) is a member of the integrin adhesion receptor family and helps platelets to attach to endothelial exposed collagen (Hoffman et al., 2005, Hoffman et al., 2008, Clemetson et al., 1999). $\alpha_2\beta_1$ mainly acts as a secondary receptor for collagen adhesion to platelets (Siljander et al., 2004). In contrast, GPVI serves primarily as a collagen receptor that directly interacts with the collagen and stimulates intracellular signaling cascades. The collagen-platelet interaction is a complicated process and the antiplatelet medications that target collagen receptor are limited to patients with high thrombotic and low bleeding risk, for a short period of time (Gachet, 2015, Franchi and Angiolillo, 2015).

ADP is one constituent of platelet dense granules that are released in response to physiological platelet agonists, including collagen, TXA2 and thrombin. ADP also amplifies its own effects as well as those of other activators (Shankar et al., 2006, Maffrand et al., 1988). In addition, ADP is released passively by damaged endothelium and red blood cells that enhance platelet aggregation via integrin $\alpha_{IIb}\beta_3$ activation and subsequent fibrinogen binding (Mills, 1996). Adding ADP to platelets *in vitro* leads to TXA2 formation, phosphorylation of PIP_2, raised cytosolic Ca^{2+} concentration, shape change, aggregation and granules release, as well as inhibition of cAMP formation (Daniel et al., 1986, Mills and Smith, 1972, Raha et al., 1993, Sage and Rink, 1986).

Platelets have two ADP-dependent purinergic receptors, P_2Y_1 and P_2Y_{12}, members of the GPCR superfamily (Jantzen et al., 1999, Daniel et al., 1998). The third platelet purinergic receptor is $P2X_1$, an ATP-gated Ca^{2+} channel (Daniel et al., 1998, MacKenzie et al., 1996). P_2Y_1 is responsible for activation of PLC, as well as the

production of diacylglycerol and IP. As a result of IP formation, Ca^{2+} is mobilised from cytosolic stores, activates protein kinase C (PKC) and causes phosphorylation of myosin light chains (Figure 1-3). These signaling events play an important role in agonist-induced platelet shape change. P_2Y_1 activation is sufficient to cause platelet shape change. However, to induce platelet aggregation both receptors need to be activated (Daniel et al., 1998, Daniel and Adelstein, 1976, MacKenzie et al., 1996). By blocking or deleting the P_2Y_1 receptor, ADP still can inhibit cAMP formation, but its ability to increase cytosolic Ca^{2+} concentration, and cause platelet shape change and aggregation, are significantly impaired. $P2Y_1^{-/-}$ knockout mice have minimal increases in bleeding time and increased survival rate after administration of ADP. However, the responses to other platelet agonists are unaffected (Leon et al., 2001, Leon et al., 1999).

ADP binds to the P_2Y_{12} receptor on the platelet surface, triggering signaling through G inhibitory protein to inhibit adenylyl cyclase and cAMP formation (Hollopeter et al., 2001, Zhang et al., 2001), leading to dephosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Figure 1-3) (Cattaneo, 2007). VASP phosphorylation is directly proportional to the degree of P_2Y_{12} antagonism (Cattaneo, 2007). Patients lacking functional P_2Y_{12}, have a mild hemorrhagic phenotype because their platelets do not aggregate normally in response to ADP (Cattaneo and Gachet, 1999, Nurden et al., 1995). In mice, deletion of either P_2Y_1 or P_2Y_{12} impairs the response of platelets to low concentrations of thrombin, TXA2 and ADP, and prolongs bleeding time (Leon et al., 1999, Fabre et al., 1999).

1.2.1.3. Granules

Activation of platelets by ADP, collagen and thrombin trigger morphological changes in platelets that result in the movement of secretory granules to the cell centre and consequent fusion with the surface-connected canalicular system. As a result of granule-plasma membrane fusion, platelets release their granule components (Siess, 1989, Stenberg et al., 1984, Escolar and White, 1991). Platelets are full of secretory granules including α granules, dense granules, lysosomes and peroxisomes, which are important for platelet function (Figure 1-1) (Coppinger et al., 2004).

The most abundant secretory granules in platelets are α-granules, forming approximately ~10% of platelet volume (Table 1-1). In addition, there are around 50-80 α-granules per platelet with sizes ranging from 200-500 nm (Frojmovic and Milton, 1982). Platelet α-granules play roles in angiogenesis, antimicrobial activity, wound healing, atherosclerosis, inflammation, host defense, and malignancy (Blair and Flaumenhaft, 2009).

Dense granules contain small particles, including calcium ions and serotonin, as well as nucleotides, including ADP and adenosine triphosphate (ATP). These contents promote localised vasoconstriction and activate other platelets so any change in granules release may affect normal haemostasis (Ren et al., 2008). Platelet peroxisomes and lysosomes contain catalase and hydrolytic enzymes such as cathepsins and hexosaminidase, which might play a role in platelet activation and clot remodeling (Ren et al., 2008, Kamath et al., 2001, Anitua et al., 2004). Each of these secretion events should be highly controlled to avoid the inappropriate release of granules contents.

1.2.1.4. Microparticles

Microparticles are heterogeneous, membrane-coated small vesicles with a diameter of 0.1-1 μm that are released from different cell types, including platelets. Microparticles contain a variety of intracellular components such as DNA, RNA and cytoplasmic proteins, and express proteins derived from their original cell source. They are released from plasma membranes by a process called 'exocytic budding' following cell activation or apoptosis. Formation of microparticles is an active process, being constantly produced by cells. In this process, the normal asymmetry of membrane lipids is misplaced, which causes phosphatidylserine to be on the outer layer of the microparticle membrane. As a result, annexin V, which is commonly used to detect and quantify microparticles, is allowed to bind to phosphatidylserine (Distler et al., 2005, Wolf, 1967). Several markers have been used to determine the origins of microparticles such as platelets (CD41a/b, CD42a/b and CD61), endothelial cells (CD144, CD105, CD146, CD51, CD62E AND CD31+/CD42a-) and erythrocytes (CD235a) (Gelderman and Simak, 2008, Jimenez et al., 2003, Jy et al., 2004).

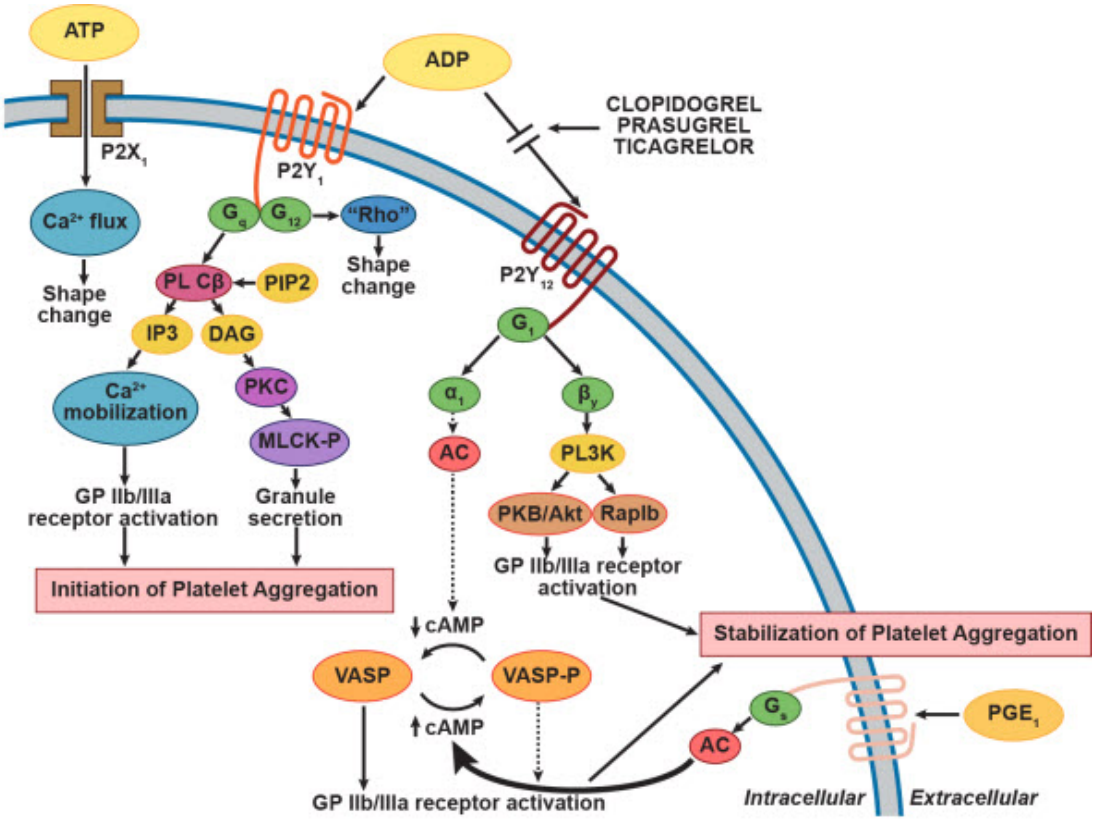

Figure 1-3 ADP pathway in platelet aggregation.

Activation of the P_2Y_1 receptor leads to an alteration in shape and initiates a weak and transient phase of platelet aggregation. The binding of ADP to the Gq-coupled P_2Y_1 receptor activates phospholipase C (PLC), which generates diacylglycerol (DAG) and inositol triphosphate (IP3) from phosphatidylinositol biphosphate (PIP2). Diacylglycerol activates protein kinase C (PKC) leading to phosphorylation of myosin light chain kinase (MLCK-P) and IP3 leads to mobilisation of intracellular calcium. The P_2Y_1 receptor is coupled to another G-protein, G12, which activates the "Rho" protein and leads to the change in platelet shape. The binding of ADP to the Gi-coupled P_2Y_{12} receptor liberates the Gi protein subunits α_i. β and γ, resulting in stabilisation of platelet aggregation. The α_i subunit inhibits adenylyl cyclase (AC) and, thus, reduces cyclic adenosine monophosphate (cAMP) levels, which diminishes cAMP-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP-P). The status of VASP-P modulates glycoprotein (GP) IIb/IIIa receptor activation. The subunit β_γ activates the phosphatidylinositol 3-kinase (PI3K), which leads to GP IIb/IIIa receptor activation through activation of a serine-threonine protein kinase B (PKB/Akt) and of Rap1b GTP binding proteins. Prostaglandin E1 (PGE1) activates AC, which increases cAMP levels and status of VASP-P. Solid arrows indicate activation; dotted arrows indicate inhibition. Image sourced from (Angiolillo et al., 2007) without modifications.

Table 1-1 Platelet Alpha Granules Contents and Functions

Functions	Contents	References
Chemokines	CXCL1 (GRO-α), CXCL4 (platelet factor 4), CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES	(Brandt et al., 2000, Gleissner et al., 2008)
Growth factors	Vascular endothelium growth factor (VEGF), Platelet-derived growth factor (PDGF), Fibroblast growth factor (FGF), Epidermal growth factor (EGF), Hepatocyte growth factor (HGF), Insulin-like growth factor (IGF)	(Rendu and Brohard-Bohn, 2001, Nurden et al., 2008)
Angiogenesis inhibitors	TSP-1	(Jimenez et al., 2000)
Pro angiogenic mediators	Angiopoietin, CXCL12 (SDF-1α), Matrix metalloproteinases (MMP-1, -2, and -9)	(Karshovska et al., 2007, Massberg et al., 2006)
Complement and complement binding proteins	Complement C3, Complement C4 precursor	(Maynard et al., 2007)
Adhesive receptors	GPIbα-IX-V (major receptor for fibrinogen), Integrin αIIbβ3, GPVI (collagen receptor)	(Berger et al., 1996, Suzuki et al., 2003)
Membrane bound proteins	Integrins (e.g., α_{IIb}, α_6, β_3), Immunoglobulin family receptors (e.g. GPVI, Fc receptors, PECAM), Leucine-rich repeat family receptors (e.g., GPIb-IX-V complex), Tetraspanins (e.g., CD9), Other receptors (CD36, Glut-3), P-selectin	(Suzuki et al., 2003, Nurden et al., 2004, Niiya et al., 1987, Maynard et al., 2007, Berger et al., 1993)

Coagulation factors and co-factors	Factors V, Factors XI, Factor XIII	(Rendu and Brohard-Bohn, 2001, Hayward et al., 1995, Jeimy et al., 2008, Kiesselbach and Wagner, 1972)
Inactive precursor	Thrombin, Prothrombin, High molecular weight kininogens, Plasminogen	(Maynard et al., 2007, Rendu and Brohard-Bohn, 2001)
Inhibitory proteases	Plasminogen activator inhibitor-1 (PAI-1), α_2-antiplasmin	(Rendu and Brohard-Bohn, 2001)
Anticoagulants	Antithrombin, C1-inhibitor	(Blair and Flaumenhaft, 2009)
Adhesion molecules	Fibrinogen, Von Willebrand factor (vWF), Fibronectin, Vitronectin, Thrombospondin	(Cramer et al., 1988, Gralnick et al., 1985)

Platelet-derived microparticles (PMP) represent subcellular elements for signaling between cells, and intercellular communication in inflammation. PMP are able to trigger coagulation *in vitro* due to the expression of tissue factor on their surface (Shimura et al., 1996, Schecter et al., 2000, Nieuwland et al., 1997, Joop et al., 2001), and by providing a negatively charged surface (phosphatidylserine) for the prothrombinase complex (factors Va and FXa) to assemble (Gilbert et al., 1991, Hamilton et al., 1990, Hoffman et al., 1992). The prothrombinase complex catalyses the conversion of the inactive zymogen prothrombin to the enzyme thrombin. Furthermore, tissue factor (the trigger of the tissue factor pathway) and vWF multimers (which facilitates platelet aggregation), have been detected on circulating PMP (Morel et al., 2011b). Moreover, PMP promote cyclooxygenase-2 (COX-2) expression in endothelial cells, which leads to an increase in prostaglandin production and vasodilation (Barry et al., 1997, Barry et al., 1998).

Microparticles from different origins have been proposed as future prognostic, diagnostic or predictive biomarkers for several diseases, as they change composition, phenotype and number in a variety of pathological conditions (Gyorgy et al., 2011, Ramacciotti et al., 2010, Rautou et al., 2011, van der Zee et al., 2006). Increased levels of circulating PMP are associated with prothrombotic diseases such as myocardial infarction (van der Zee et al., 2006), sickle cell anemia (Shet et al., 2003), heparin-induced thrombocytopenia (Hughes et al., 2000) and inflammation (Daniel et al., 2006, Pereira et al., 2006, Pamuk et al., 2006). In prototypic autoimmune diseases such as SLE, it has been shown that SLE patients have elevated levels of PMP, which are associated with an increased potential for thrombin generation (Pereira et al., 2006). As microparticles contain DNA, or RNA, or both, it

has been suggested that they might act as autoadjuvants to activate macrophages, lymphocytes and dendritic cells (DC), stimulate cytokine and chemokine production (Piccin et al., 2007, Ratajczak et al., 2006, Beyer and Pisetsky, 2010). In rheumatoid arthritis, microparticles appear to contribute directly to synovial inflammation, and recently it has been found that PMP in synovial fluid might exacerbate arthritis (Berckmans et al., 2002, Boilard et al., 2010).

1.2.2. The Role of Platelets in Atherosclerosis

Atherosclerosis is a systemic inflammatory disease influenced by blood cells, including platelets, which accumulate in the intima of large and medium-sized arteries (Ross, 1999, Lusis, 2000). Injury to the endothelium causes endothelial dysfunction that changes the normal homeostatic properties of the endothelium, from an anticoagulant to procoagulant, releasing growth factors, cytokines and vasoactive molecules. Atherosclerotic lesions begin to grow and progress on dysfunctional endothelium.

Platelets mediate atherosclerotic lesion formation by releasing their granule contents and microparticles following adhesion and activation, in addition to forming free AA that can be converted into TXA2 or leukotrienes. TXA2 is a potent platelet agonist and vasoconstricting agent, while leukotrienes amplify the inflammatory response (Ross, 1999). Indeed, it has been reported that platelet-derived proinflammatory factor deposition on monocytes and on the vascular wall, lead to increased monocyte recruitment, activation of monocyte integrins and exacerbation of atherosclerotic lesions (Huo et al., 2003, Chesterman and Berndt, 1986). In addition, inhibition of platelet adhesion decreases leukocyte infiltration and weakens the development of atherosclerotic plaques in the cholesterol-fed apolipoprotein E-deficient ($ApoE^{-/-}$)

mouse (Huo et al., 2003). In conclusion, the contents released from platelet granules during the release reaction play a major role in the development of atherosclerosis.

1.3. Cannabinoid Receptors

Cannabinoid (CB) receptors are so named as they are activated by cannabinoids such as delta-9-tetrahydrocannabinol (THC) (Howlett et al., 1988). The two CB receptors described to date, CB1 and CB2, are different in their predicted amino acid sequence and signaling mechanisms. Both however, are coupled through G protein (GPCRs) to adenylyl cyclase and mitogen-activated protein kinase (Rueda et al., 2000).

CB1 receptors are present mainly in central and peripheral neurons, with a major function to inhibit neurotransmitter release (Childers and Deadwyler, 1996, Pertwee, 2001). In contrast, the CB2 receptor is predominantly expressed on immune cells and affects many functions of the immune response, including modulation of cytokine release (Zhu et al., 2000). Platelets express CB1, and to lesser extent CB2, receptors, although their roles in platelet function are unclear (Deusch et al., 2004, Catani et al., 2010).

1.3.1. Cannabinoid Receptors in Disease

The CB system is able to modulate immune function through the CB2 receptor that is expressed by macrophages, NK cells, T cells and B cells, in addition to its important role in neuronal regulation through the CB1 receptor (Klein et al., 2003). In the healthy central nervous system (CNS), CB1 and CB2 receptors are normally undetectable (Buckley et al., 2000, Galiegue et al., 1995, Munro et al., 1993). However, CB1 and CB2 expression changes in relation to cell activation (Carlisle et al., 2002, Garcia-Ovejero et al., 2009). For example, CB2 receptor expression is

provoked in the spinal cord, and in chronic pain models related to peripheral nerve injury, which are associated with the presence of activated microglial cells (Zhang et al., 2003).

Neuritic plaque-associated astrocytes and microglia in patients with Alzheimer's disease contain activated microglial cells with raised CB2 receptor expression, whereas the expression of CB1 receptors remains unchanged (Benito et al., 2003). It has been found that the cannabinoid system and microglial cells are also associated with CNS inflammation in multiple sclerosis (Compston and Coles, 2002). Furthermore, the expression of CB2 receptors in CNS is dramatically upregulated in experimental autoimmune encephalomyelitis associated with infiltrating macrophages and resident microglia (Ponomarev et al., 2005). It has been reported that low doses of THC act through CB2 receptors to reduce the progression of atherosclerotic plaques in a murine knockout model of atherogenesis, via suppression of macrophage recruitment (Steffens et al., 2005). In any case, the selective presence of cannabinoid receptors in different human cells suggest that the modulation of their activity may have potential therapeutic implications (Pertwee, 2006).

1.3.2. Endogenous Cannabinoids (Endocannabinoids)

Endocannabinoids are AA derivatives and part of the eicosanoid family (Blankman et al., 2007). They are the endogenous ligands for CB receptors and a part of endocannabinoid system as well as the enzymes for ligand biosynthesis and degradation for molecules, such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (Freund et al., 2003, Mackie, 2006).

The main endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide (AEA) activate CB receptors, and in the case of AEA, TRPV1 channels (Figure 1-4). Moreover, it has been reported that platelets are able to metabolise 2-AG and AEA (Maccarrone et al., 1999, MacCarrone et al., 2001, Braud et al., 2000, Gkini et al., 2009, Varga et al., 1998). Both 2-AG and AEA act as platelet agonists however, the literature relating to the actions of endocannabinoids in platelets is controversial. It has been reported that 2-AG activates human platelets through CB1 and CB2 receptors, as blocking these receptors inhibited the 2-AG stimulatory effect on platelets (MacCarrone et al., 2001). In contrast, another study reported that 2-AG induced platelet aggregation through a CB1- and CB2-independent mechanism (Keown et al., 2010). The reasons for these conflicting findings are unknown, however, it might be due to using different experimental models e.g., platelet rich plasma (PRP) versus whole blood in aggregation studies, as well as different CB1 and CB2 receptor antagonists.

2-AG and AEA hydrolysis generates AA, which is the major substrate of cyclooxygenase (Nakahata, 2008). It has been reported that AEA activates platelets from rabbits, but this was insensitive to CB1 antagonist and inhibited completely by a COX1 inhibitor (Braud et al., 2000). Another study has shown the same effect using human platelets. However, inhibiting COX1 did not have an effect on platelet activation suggesting that AEA activation may be mediated through cannabinoid receptor-dependent mechanisms (Maccarrone et al., 1999).

THC activates platelets by increasing GPIIb/IIIa expression, as well as P-selectin and 2-AG levels (Deusch et al., 2004). Finally, in addition to AEA, there are other endocannabinoids that act also as an endovanilloids (discussed later in 1.6.1

Endogenous Vanilloids), such as N-oleoyldopamine (OLDA) and N-arachidonoyl-dopamine (NADA) (Figure 1-5).

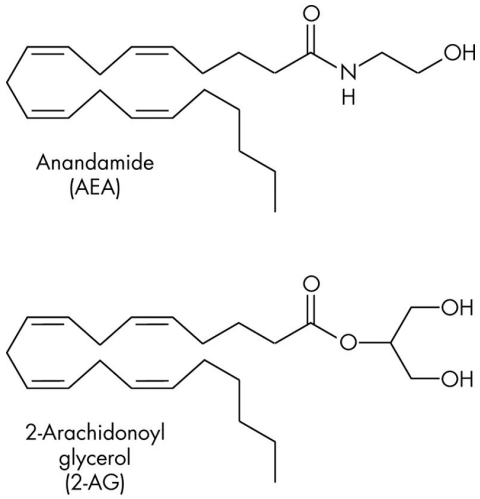

Figure 1-4 Chemical Structures of Endogenous Cannabinoids

Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) (Tomida et al., 2004).

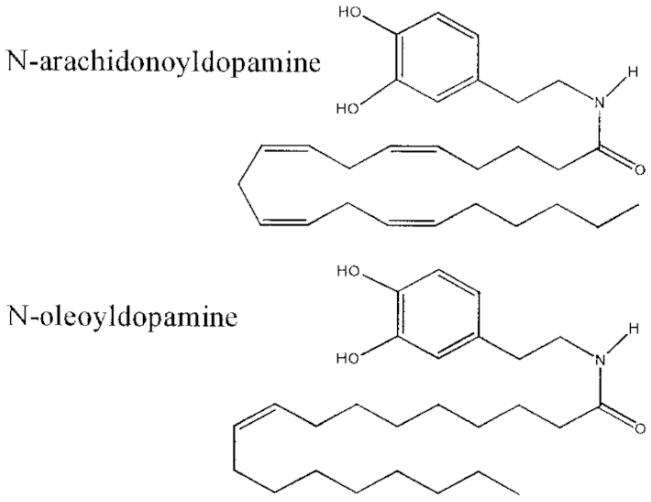

Figure 1-5 Chemical Structures of Endovanilloids/Endogenous Cannabinoids

N-oleoyldopamine (OLDA) and N-arachidonoyl-dopamine (NADA) (Chu et al., 2003).

1.4. Transient Receptor Potential Vanilloid Channel-1

The Transient Receptor Potential Vanilloid Channel-1 (TRPV1) channel, also referred to as the CAP receptor (Roberts et al., 2004), is a non-selective cation channel that is stimulated by a wide variety of agents. These include plant-derived vanilloids (e.g. CAP and resiniferatoxin (RTX)), endogenous vanilloid-like lipids (e.g. OLDA and NADA), acid (pH < 7) and heat (> 43 ^{0}C) (Caterina and Julius, 2001, Zhong and Wang, 2008). The TRPV1 channel is widely distributed throughout the peripheral nervous system, central nervous system and non-neuronal tissue (Sanchez et al., 2001).

There are several amino acids and amino acid sequence motifs in the TRPV1 channels protein that have defined functions, such as mediating the actions of CAP (Kuzhikandathil et al., 2001, Tominaga and Tominaga, 2005, Welch et al., 2000), heat activation (Gunthorpe et al., 2000), phosphorylation (Rathee et al., 2002, Dai et al., 2004), modulation by lipids (Ahern, 2003, Chuang et al., 2001), multimerisation (Smith et al., 2002), proton action (Jordt et al., 2000), permeability (Caterina et al., 1997, Mohapatra et al., 2003) and desensitisation (Szallasi and Blumberg, 1999) (Figure 1-6, describes these features in more detail).

Capsaicin is lipophilic, which allows it to pass through the cell membrane and acts on binding sites on the intracellular domain of TRPV1, providing a possible explanation for the lag time between capsaicin intake and pungent sensation (Jung et al., 1999). TRPV1 has a similar structure to voltage-gated K^+ channels, involving the six-TM topology. As specified by the contemporary helix-packing models of the voltage-gated K^+ channels, the first, second and third TM domains are placed on the lipid-facing side of the tetrameric channel complex. In contrast, the fifth and sixth

TM domains are placed nearer to the pore-forming channel core. Presuming TRPV1 is similar to helix packing, the capsaicin lipophilic moiety might bind to the second and third TM domains on the channel-lipid interface. The vanilloid moiety might interact with residues around Tyr 511 in the cytosolic region, therefore binding the cytosolic tail with two TM domains (Kuzhikandathil et al., 2001, Tominaga and Tominaga, 2005, Welch et al., 2000).

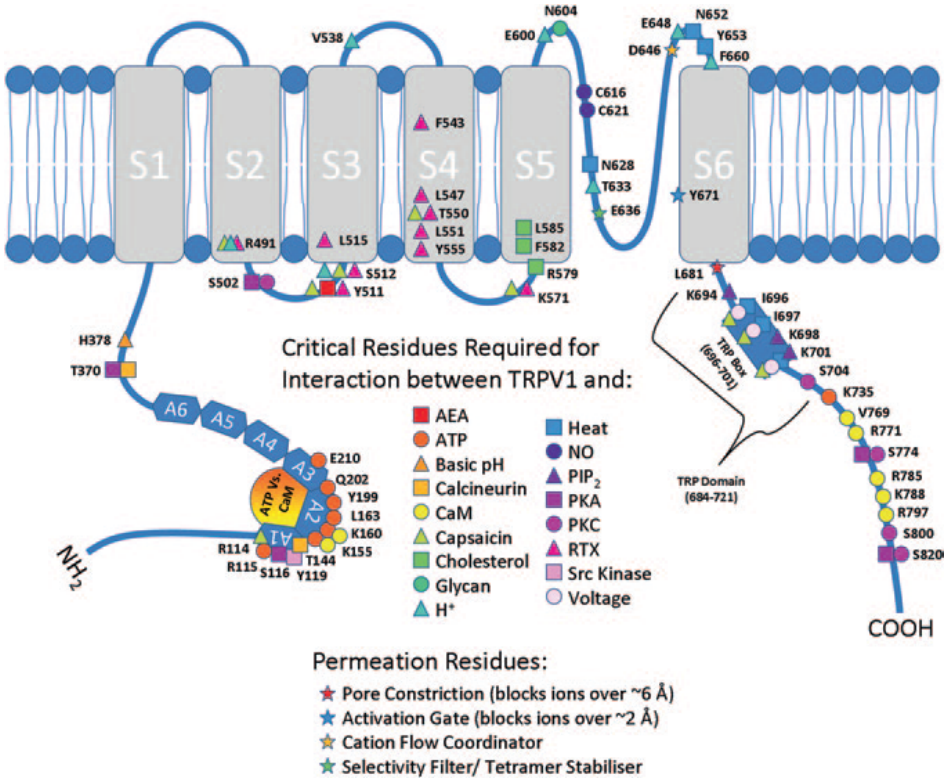

Figure 1-6 Critical residues involved in TRPV1 function.

TRPV1 has six transmembrane domains and a short, preforming hydrophobic stretch between the fifth and sixth transmembrane domains. TRPV1 topology highlighting residues implicated in the activation, sensitisation, desensitisation, gating properties or permeation of the channel. CAP passes through the cell membrane to its binding sites in the intracellular domain. Phosphatidylinositol (4,5)-bisphosphate (PIP_2) binds to the indicated region in the carboxyl (*C*) terminus. Calmodulin (*CaM*) binds to both C- and amino (*N*)-termini. Image sourced from (Nagy et al., 2014) without modifications.

1.4.1. TRPV1 in Disease

Upregulation of TRPV1 channel expression occurs on certain pathological conditions, which may provide a basis for the use TRPV1 channels antagonists as potential therapeutic agents (Cortright and Szallasi, 2004). For example, abnormal expression of TRPV1 channels by neurons that do not normally express the TRPV1 channels has been linked to the development of neuropathic pain and inflammatory hyperalgesia (Hudson et al., 2001, Rashid et al., 2003). Indeed, the expression of TRPV1 in dorsal root ganglia increased following dorsal root ganglia avulsion injury (e.g. central axotomy) (Smith et al., 2002).

A number of disorders are correlated with increased aberrant expression of TRPV1 for example: in the respiratory system, airway neurons express TRPV1 upon exposure to allergen leading to release neuropeptides, which increase airway secretions and submucosal edema, alter smooth muscles tone, and stimulate immune and inflammatory cellular responses (Bessac and Jordt, 2008, Lieu et al., 2012). In nasal mucosa allergic inflammation, mucosal expression of TRPV1 increases (Samivel et al., 2016), as do TRPV1 channel expression in mononuclear cells of end-stage kidney disease patients (Saunders et al., 2009).

The number of TRPV1-like immunoreactive fibers increases in patients' colon with active inflammatory bowel disease and irritable bowel syndrome and in a rectal biopsy from patients with fecal urgency and rectal hypersensitivity (Chan et al., 2003, Yiangou et al., 2001, Akbar et al., 2010). Furthermore, patients with neurogenic detrusor over-activity have an increased expression of TRPV1 and were

effectively treated with TRPV1 agonists, CAP or RTX (Chancellor and de Groat, 1999).

High fat fed TRPV1 knockout mice were protected against obesity-induced hypertension and glucose intolerance compared to high-fat-fed wild-type mice (Marshall et al., 2013). In summary, disease-related changes in the expression of TRPV1 have been described in human, however, the mechanism(s) regulating TRPV1 gene expression in diseased conditions are unknown. Therefore, better understanding of these mechanisms will have implications for drug development.

1.5. Vanilloids

Vanilloids are compounds that contain a vanillyl group and include endogenous agents (endovanilloids) that are primarily AA derivatives, and plant-derived vanilloids, with the most well-known being capsaicinoids, the active 'hot' constituents of chilli peppers.

1.5.1. Endogenous Vanilloids

Endogenous AA derivatives activate TRPV1 channels and CB receptors to varying degrees, and include at least three types of lipids: N-acylethanolamines [N-oleoylethanolamine, AEA, N-linoleoylethanolamine] (Zygmunt et al., 1999, Ross, 2003); unsaturated N-acyldopamines [OLDA, NADA] (Chu et al., 2003, Huang et al., 2002) and lipoxygenase products [e.g., leukotriene B_4, 12-(S)- and 15-(S)-hydroperoxyeicosatetraenoic] (Hwang et al., 2000).

NADA was originally characterised in the striatum of bovine brain, and the areas with the highest concentrations in rat nervous system include the cerebellum,

striatum and hippocampus, whereas the lowest concentration is found in the dorsal root ganglion (Huang et al., 2002). In addition, NADA has nanomolar affinity for TRPV1 channels on sensory neurons, which leads to the release of calcitonin gene-related peptide and substance P (Huang et al., 2002), and also possesses the same affinity for CB1 receptors (Bhave et al., 2002). NADA was the first endovanilloid identified in mammals with similar efficacy and potency at TRPV1 channels comparable to that of CAP (Huang et al., 2002). OLDA is the most selective and potent endogenous vanilloid at the TRPV1 channels, and might function as a central or peripheral mediator of TRPV1 channel activation. Moreover, OLDA is 50 times more effective at TRPV1 channels compared to CB1 receptors. Also it is more potent than CAP by 30 times (Chu et al., 2003, Szolcsanyi et al., 2004). Moreover, OLDA is quite stable and remains for long periods (hours) in bio-membranes, potentially activating receptors for extended periods (Zajac et al., 2006).

Importantly, NADA and OLDA inhibit *in vitro* platelet aggregation induced by ADP and collagen in a concentration-dependent manner, and both are more potent than CAP and DHC (Almaghrabi et al., 2014). Moreover, since platelets have CB1 and CB2 receptors (Deusch et al., 2004, Catani et al., 2010) and FAAH (Maccarrone et al., 1999), a prominent component of endocannabinoid system, the possibility that endocannabinoids/endovanilloids can modulate platelet function is a distinct possibility.

1.5.2. Plant Derived Vanilloids

The placental tissues of *Capsicum* fruit contain capsaicinoids (Kozukue et al., 2005, Thompson et al., 2005a, Thompson et al., 2005b, Garces-Claver et al., 2006). CAP and DHC are the two major capsaicinoids that are responsible for the ‘pungency’ of

chilli peppers (Bennett and Kirby, 1968, Kosuge and Furuta, 1970). The relative proportions of CAP:DHC in many common pepper varieties is 60:40 (Garces-Claver et al., 2006). Besides CAP and DHC, at least nine other capsaicinoids have been found to form in peppers including, norcapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, homocapsaicin II, homodihydrocapsaicin II, N-vanillyl nonanamide, N-vanillyl octanamide, and N-vanillyl decanamide (Reilly et al., 2001a, Reilly et al., 2001b, Davis et al., 2007, Mueller-Seitz et al., 2008). Capsaicinoids are synthesised by condensation of vanillylamine and fatty acids, mainly in the pepper placenta (Thiele et al., 2008, Curry et al., 1999), are stable in nonpolar and polar solvents, and highly lipophilic (Tanaka et al., 2009).

It is well known that capsaicinoids exert their actions by stimulating TRPV1 channels, and the mechanism is now well described (Luo et al., 2011). In the nervous system, capsaicinoids act as a natural irritant and selectively activate TRPV1 channels on sensory neurons, which convey noxious sensations to the central nervous system. Activated TRPV1 channels allow sodium and calcium ions to move through the sensory neuron membrane, resulting in depolarisation and nociceptive responses. These are followed by a long-lasting refractory period and desensitisation of the neuron (Wang and Woolf, 2005, Gerner et al., 2008, Kissin, 2008, Knotkova et al., 2008). Each analog binds to and activates TRPV1 channels with different potency, and is dependent on a 3-methxy-4-hydroxybenzylamine ring (vanilloid) and alkyl chain structure (Hayes et al., 1984, Walpole et al., 1993a, Walpole et al., 1993c, Walpole et al., 1993b). CAP, nonivamide and DHC are the most pungent and potent vanilloids in *Capsicum* (Reilly and Yost, 2006).

1.5.3. Clinical Applications of Plant-Derived Vanilloids

Capsaicinoids have extensive pharmacological properties, however their clinical application is to date limited by their high toxicity and low selectivity (Luo et al., 2011). Some examples of the physiological and pharmacological effects of plant-derived vanilloids, and their potential clinical applications, are discussed below.

As an analgesic, administration of CAP locally or orally reduces pain in rheumatoid arthritis, inflammatory heat, noxious chemical hyperalgesia, and neuropathic pain (Fraenkel et al., 2004, Raber et al., 2015). In oncology studies, CAP has been shown to reduce the size of MDAMB231 breast cancer masses in mice (Thoennissen et al., 2010) and was able to destroy cultured prostate cancer cells, while DHC was reported to enhance autophagy in cultured human HCT116 colon cancer cells (Oh et al., 2008, Thoennissen et al., 2010, Yang et al., 2010). Moreover, it has been found in clinical studies and animal experiments that capsaicinoids suppress obesity by decreasing body fat accumulation (Shin and Moritani, 2007, Reinbach et al., 2009). Capsaicinoids have also gastroprotective effects in various animal models, where gastric ulcers were induced by hydrochloric acid, aspirin, ammonia, indomethacin or ethanol (Szolcsanyi and Bartho, 2001, Mozsik et al., 2007).

CVD such as atherosclerosis, myocardial infarction and coronary heart disease are associated with significant morbidity and mortality, and there is growing evidence that capsaicinoids have many beneficial effects on the cardiovascular system (Harada and Okajima, 2009, Peng and Li, 2010). CAP-sensitive sensory nerves that are rich in TRPV1 channels are densely distributed in the cardiovascular system. Activation of TRPV1 channels by exogenous agonists has been reported to exert hypotensive effects and protective effects against cardiac injury by stimulating the formation and

release of neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P (Zvara et al., 2006, Peng and Li, 2010). Thus, TRPV1 channels are a target for drug development and therapy to treat CVD.

It is believed that low-density lipoprotein (LDL) oxidation is the initiating factor for the growth and progression of atherosclerotic plaques. Serum total cholesterol and lipid peroxide levels in high fat fed rats are reduced following CAP treatment, in an *in vivo* study (Manjunatha and Srinivasan, 2006, Manjunatha and Srinivasan, 2007). In addition, it has been found that CAP and DHC were able to raise LDL resistance to oxidation *in vitro* by slowing the initiation of oxidation and/or delaying the oxidation rate (Ahuja et al., 2006a). Regular consumption of chilli for four weeks in humans increased the resistance of serum lipoproteins to oxidation (Ahuja and Ball, 2006) as well as reducing hyperinsulinaemia and hyperglycaemia (Ahuja et al., 2006b, Chaiyasit et al., 2009). Indeed, a recent large prospective cohort study in China (~500,000 participants), reported that regular spice intake significantly reduced all-cause mortality, in particular, ischemic heart disease and diabetes, which was attributed primarily to chilli constituents (Lv et al., 2015). Finally, the antioxidant property of capsaicinoids makes them a useful preventive tool for CVD and in particular atherosclerosis and coronary heart diseases (Luo et al., 2011). Although the clinical application of CAP has been restricted to pain management, these other observations support further research into additional uses for CAP and other vanilloids in cancer, obesity, CVD and gastrointestinal ulcers.

1.5.4. Toxicity of Plant-Derived Vanilloids

The desensitising and cytotoxic properties of capsaicinoids have been well documented in peripheral sensory neurons (Aδ and C-fiber) and applied for chronic pain treatment (Szallasi and Blumberg, 1999, Wood et al., 1988). Furthermore, it has been found that activation of TRPV1 channels expressed by cultured neurons isolated from rat dorsal root ganglia enhanced cell death (Szallasi and Blumberg, 1999). In neurogenic inflammation, CAP stimulates TRPV1 channels- and calcium-dependent release of substance P, and neuropeptides from the airway tissue neurons to promote inflammatory responses to potentially harmful stimuli (Veronesi and Oortgiesen, 2006, Veronesi et al., 1999). Therefore, TRPV1 antagonists might be the future treatment for respiratory inflammation.

Capsaicinoids are known to cause coughing, severe irritation and respiratory inflammation in experimental human and animal models. This has been demonstrated through studies that have demonstrated that CAP and neuropeptide synergistically enhanced the production of inflammatory mediators (tumor necrosis factor, interlukin-6 and -8) from normal human bronchiolar epithelial cells, human bronchiolar epithelial cells (BEAS-2B), and the human lung adenocarcinoma cell line (A549) (Quay et al., 1998, Veronesi et al., 1999, Veronesi et al., 2000). Furthermore, activation of TRPV1 channels on respiratory epithelial cells by capsaicinoids initiates the production of pro-inflammatory cytokines that promote host defense responses and may lead to cell death. Capsaicin-induced cell death was greater in the BEAS-2B and A549 cell-lines with high TRPV1 channels expression, suggesting that TRPV1 channels might be the key mediator of CAP effect (Reilly et

al., 2003). Finally, CAP might have a potential role in cancer prevention (Yang et al., 2010, Surh, 2002, Oh et al., 2008).

1.6. Vanilloids and Platelets

This section discusses the studies that have investigated the effects of vanilloids on *in vivo* and *in vitro* platelet aggregation, and their potential mechanism/s of action (Table-1-2). It has been found that plant-derived vanilloids, CAP and DHC, as well as endovanilloids, OLDA and NADA, inhibited *in vitro* ADP-induced platelet aggregation in a concentration-dependent manner (Almaghrabi et al., 2014). The effects of both classes of vanilloids on aggregation induced by ADP were not shown to be TRPV1-dependent or through the destruction of platelets (Almaghrabi et al., 2014). Additionally, plant-derived vanilloids inhibited AA-induced aggregation while endovanilloids inhibited collagen-induced platelet aggregation (Almaghrabi et al., 2014). A study using canine platelets reported that CAP inhibited *in vitro* platelet aggregation induced by collagen in a concentration-dependent manner, but this inhibition was not mediated through TRPV1 channels as A-993610, the TRPV1 antagonist, had no effect (Mittelstadt et al., 2012).

Adams et al. (2009) found that CAP inhibits *in vitro* platelet aggregation in a concentration-dependent manner, while DHC exhibits a bimodal effect on platelet aggregation, stimulating at 3.125 μM, and inhibiting from 25-100 μM. Furthermore, CAP inhibited *in vitro* platelet aggregation induced by AA dramatically compared to other agonists, ADP, calcium ionophore and collagen, through inhibition of the COX1 enzyme in platelets (Raghavendra and Naidu, 2009). Previous studies suggested that the anti-platelet effect of CAP was not through specific membrane

receptors, but through the insertion into the plasma membrane of platelets, changing membrane their ionic permeability and/or fluidity (Hogaboam and Wallace, 1991).

In contrast to previous studies, Sandor et al., (2014), examined the effect of oral administration of 400 μg CAP + 500 mg aspirin and 800 μg CAP + 500 mg aspirin on 15 healthy males, compared to aspirin and CAP alone, in the first clinical human trial on the effect of CAP on platelet aggregation *in vivo*. Blood was drawn before and after administration of the drugs, platelet aggregation was then induced by adrenaline and measured using optical aggregometry. The authors showed that oral CAP did not influence the inhibitory effect of aspirin on platelet aggregation induced by adrenaline, compared to aspirin alone. Furthermore, CAP (400 and 800 μg) by itself did not have any effect on *in vitro* platelet aggregation (Sandor et al., 2014).

Harper et al. (2009) reported the presence of TRPV1 channel in human platelets, and that CAP is able to induce a concentration-dependent increase in Ca^{2+} release and Ca^{2+} influx from intracellular platelets stores, that was suppressed by different TRPV1 antagonists (5-iodo-resiniferatoxin and AMG 9810) (Harper et al., 2009). Furthermore, Harper et al. (2009) has suggested that TRPV1 contributes to the activation of platelets induced by ADP and thrombin by promoting endovanilloids formation in response to platelets agonists. These findings are in conflict with other recent and earlier studies, which demonstrated that capsaicinoids inhibit, rather than activate, platelet aggregation (Adams et al., 2009, Almaghrabi et al., 2014, Mittelstadt et al., 2012, Raghavendra and Naidu, 2009, Hogaboam and Wallace, 1991).

The reasons for the discrepant results across these studies are not known, however, it may be due to a variety of reasons such as; species, as platelets from various species have different sensitivity to several platelets agonists (Nylander et al., 2006), and the concentrations of platelet agonists employed, as the magnitude of aggregation response is concentration-dependent (Maayani et al., 2003). In addition, there were differences in incubation times. The anti-haemostatic effects of capsaicinoids may potentially prevent and/or decrease the incidence of CVD risk. However, the exact mechanism(s) responsible for the effect of vanilloid-like agents on platelet aggregation are not well understood, and their effect on platelets from patients with high risk of thrombosis and atherosclerosis such as SLE patients, is unknown.

Table 1-2 Summary of studies on the effects of vanilloid/s on platelet aggregation in reverse chronological order

Studies	Origin of platelets	Agonist/s	Incubation time	Vanilloid/s and/or endovanilloids	The effect/s on platelet aggregation	Mechanism/s of action
Sandor et al. (2014)	Human	Epinephrine (10 μM)		400 μg CAP + 500 mg aspirin (ASA) 800 μg CAP + 500 mg ASA	Oral CAP did not influence the inhibitory effect of ASA on platelet aggregation induced by epinephrine compared to ASA alone	
Almaghrabi et al. (2014)	Human	ADP (5 and 10 μM) Collagen (4 and 8 μg/mL) AA (300 and 400 μg/mL)	No incubation	3.125, 6.25, 12.5, 25, 50 and 100 μM of CAP, DHC, OLDA and NADA, dissolved in Ethanol	OLDA and NADA completely inhibited ADP-induced aggregation CAP and DHC inhibited ADP-induced aggregation but to a lesser extent NADA & OLDA inhibited the aggregation induced by 4ug/mL collagen CAP & DHC completely inhibited AA-induced aggregation and NADA too but not to the same extend	The effect of CAP & OLDA on ADP-induced aggregation are not TRPV1 mediated TRPV1 antagonist is SB452533 CAP, DHC, OLDA and NADA are not cytotoxic to the platelets Ethanol alone has no effect on platelet aggregation
Mittelstadt et al. (2012)	Dog	Collagen (2 μg/mL)	3 minutes incubation	3, 10 and 30 μg/mL of CAP	CAP inhibited collagen-induced platelet aggregation in a concentration dependent manner	The effect of CAP on collagen-induced aggregation are not TRPV1 mediated TRPV1 antagonist is A-993610
Adams et al. (2009)	Human	ADP (5 μM)	No incubation	25, 50 and 100 μM of CAP and DHC, dissolved in Ethanol	CAP inhibited ADP-induced platelet aggregation induced by ADP in a concentration dependent manner. DHC has bimodal effect, low concentration of DHC enhanced the aggregation, while higher concentration inhibited the aggregation	The effects of CAP and DHC are unlikely to be mediated through interference with ADP receptors, as the platelets shape did not change after the exposure to CAP and DHC
* Harper et al. (2009)	Human	ADP (50 μM) Thrombin (0.5 U mL^{-1})	Five seconds	100 μM of CAP	CAP evoked platelet aggregation, a response that was inhibited in a concentration dependent manner by 5'-iodo-RTX (TRPV1 antagonist) CAP (100 μM) evoked a concentration dependent manner increase in $[Ca^{2+}]_i$ in fura-2-loaded platelets	TRPV1 is present in human platelets and CAP enhances platelet aggregation through TRPV1 channel

Raghavendra et al. (2009)	Human	ADP (50 µM) Collagen (0.5 mg/mL) AA (1 mM)	5 minutes incubation	100 and 200 µM CAP, dissolved in DMSO.	CAP inhibited AA-induced platelet aggregation in a concentration dependent manner, complete inhibition was shown with 60 µM CAP 100 µM CAP completely inhibited collagen-induced platelet aggregation 200 µM CAP inhibited ADP-induced aggregation by 58% DMSO did not show any effect on platelet aggregation	15 µM CAP inhibited TXB2 formation by 70%, using HPLC method
Hogaboam et al. (1991)	Rabbits	Thrombin (60-100 mU/mL) Calcium ionophore (1-5 µM) Platelets-activating factor (35-200 pg/mL)	Incubated	10-320 µM of CAP, dissolved in DMSO	CAP inhibited thrombin-, calcium ionophore- and platelets-activating factor-induced aggregation in a concentration dependent	CAP did not have any effect on the shape change of the platelets in response to thrombin. The ability of lipophilic molecule to alter the fluidity of the plasma membrane, and allowing calcium to stream into the neuron. Any change any platelets fluidity leads to inhibition of aggregation induced by ADP or thrombin

All the studies have used Platelet Aggregometry (PRP) to measure the effects of vanilloid/s on platelet aggregation except * Harper et al. (2009) study used $[Ca^{2+}]_i$ influx.

1.7. Systemic Lupus Erythematosus (SLE)

SLE is a multisystem autoimmune and chronic inflammatory disorder affecting mainly women of childbearing age. Patients with SLE can present with a wide array of clinical manifestations, including malar rash, polyarthritis, nephropathy, in addition to haematological manifestations (Gaubitz, 2006). SLE patients have a significantly increased risk of venous and arterial thrombosis (Ruiz-Irastorza et al., 2001). Moreover, SLE patients exhibit a high tendency to develop accelerated atherosclerosis that may lead to premature CVD (Ruiz-Irastorza et al., 2001, Salmon and Roman, 2001). Indeed, the incidence of myocardial infarction in SLE is 50-fold higher compared to sex- and age-matched healthy individuals (Manzi et al., 1997). Cardiovascular events, infection, SLE-associated organ damage, and disease activity are the main causes of mortality in SLE patients (Jacobsen et al., 1999). The treatment approach for SLE patients depends on signs, symptoms and severity of the disease. Common medications used in the treatment of SLE are: non-steroidal anti-inflammatory drugs, anti-malarial medications, steroids, immunosuppressive agents and monoclonal antibodies (Maidhof and Hilas, 2012).

1.7.1. Pathophysiology and Hematological Manifestations of SLE

Pathogenic autoantibodies are the main cause of tissue damage in SLE. Anti-double-stranded DNA (dsDNA) antibodies are thought to be the most important factors in the pathogenesis of SLE (Ter Borg et al., 1990, Isenberg et al., 2007). However, these antibodies are now considered to be one of many other SLE autoantibodies, and they are useful in the disease confirmation in the clinical setting when SLE is the

most likely diagnosis. Moreover, anti-double-stranded DNA antibodies have limited value in disease activity monitoring and flare up prediction (Fu et al., 2015).

Interferon alpha (INFα) plays an important role in the progression of SLE as it stimulates DC differentiation (Blanco et al., 2001, Banchereau and Pascual, 2006). The activation of DC leads to the release of autoantibodies against nuclear antigens and DNA, as well as stimulating B and T cells (Blanco et al., 2001, Banchereau and Steinman, 1998, Chan et al., 2012, Duffau et al., 2010).

Activated T cells initially express CD40L, a type II transmembrane protein and ligand for CD40, which induces dendritic-cell and B-cell activation. The soluble form of CD40L (sCD40L) is abundant in the serum from SLE patients and reflects disease activity (Goules et al., 2006). The main sources of sCD40L are platelets and PMP, and sCD40L has the ability to stimulate antigen-presenting cells such as DC (Elzey et al., 2003, Mehling et al., 2001, Nomura et al., 2012, Solanilla et al., 2005, Jurk and Kehrel, 2005). Serum from SLE patients has been shown to induce platelet activation to a greater extent compared to serum from healthy individuals, suggesting that sCD40L is responsible for this activation (Duffau et al., 2010).

There is evidence to support that immune complexes (DNA-anti-DNA and/or RNP-anti-RNP) are responsible for platelet activation via the FcγRIIA receptor (Duffau et al., 2010). As a result, activated platelets and DC enhance IFN-α secretion. In platelets isolated from SLE patients IFN-α controls up-regulation of multiple mRNA and proteins, and thus the modulation of platelet and megakaryocyte activity (Lood et al., 2010). Antibody production is a complicated process that involves key factors of the immune system, and these findings demonstrate the important role of IFN-α in

SLE and show that platelets can directly propagate the SLE inflammatory response (Boilard et al., 2012, Rahman and Isenberg, 2008).

Hematological problems are common in SLE patients and can affect both blood cells and the coagulation pathway, either due to the disease course itself and/or by the side effects from medications. The major manifestations include thrombocytopaenia, anaemia, and leucopaenia (Harvey et al., 1954, Howe and Lynch, 1987, Kaplan et al., 1987, Harris et al., 1985). Despite thrombocytopaenia, SLE patients have a higher risk of developing thrombosis, which further increases in the presence of antiphospholipid antibodies (APL) (West and Johnson, 1988). Anaemia occurs frequently due to chronic disease and autoimmune haemolytic anaemia (Dubois and Tuffanelli, 1964).

1.7.2. Role of Platelets in SLE

Platelets have a proinflammatory role in atherosclerosis as they release inflammatory mediators into the local environment upon activation, and change the adhesive and chemotactic properties of endothelial cells (Gawaz et al., 2005). Activated platelets may affect the development of atherosclerotic plaques by releasing adhesive ligands such as P-selectin, which are expressed on platelet membranes to mediate endothelium-platelet interactions (Celi et al., 1997). P-selectin signaling stimulates macrophages and monocytes to release growth factors and chemoattractants (Ruggeri, 2002).

Platelets are highly effective immune cells that mediate a wide range of immune responses. In addition, CD40L is highly expressed by platelets and PMP, and it has the ability to activate antigen-presenting cells, such as DC (Mehling et al., 2001,

Nomura et al., 2012, Solanilla et al., 2005, Elzey et al., 2003, Mobarrez et al., 2016, Jurk and Kehrel, 2005). In SLE, sCD40L levels are high, which leads to activation of DC and autoantibody production (Figure 1-7) (Desai-Mehta et al., 1996, Goules et al., 2006). Therefore, platelets might be a new active player in the pathogenesis of SLE through CD40L (Duffau et al., 2010, Mobarrez et al., 2016, Boilard et al., 2012).

There is significant evidence of platelet activation in SLE, as reflected by increased circulating levels of PMP, platelet and/or leukocyte aggregates, surface and soluble P-selectin, and TXB2 (Ferro et al., 1999, Nagahama et al., 2001, Tam et al., 2003, Joseph et al., 2001, Sellam et al., 2009). Finally, complement could also contribute to platelet activation in SLE, as the accumulation of complement fragment C4d and inflammatory cytokines on the platelet surface, has been reported (Gori et al., 2009, Batal et al., 2012, Navratil et al., 2006, Peerschke et al., 2010).

Antiplatelet medications are effective in the prevention of thrombotic events (Vandvik et al., 2012), with aspirin prescribed to SLE patients for primary prophylaxis of thrombosis (Arnaud et al., 2015, Arnaud et al., 2014). However, aspirin use is associated with many side effects, such as increased gastric injury and bleeding (Singh and Triadafilopoulos, 1999, Derry and Loke, 2000, Ivey et al., 1980, Weil et al., 1995). As stated earlier, studies on healthy humans, dogs and rabbits platelets have shown that the naturally occurring vanilloid, CAP, can inhibit *in vitro* platelet aggregation (Raghavendra and Naidu, 2009, Mittelstadt et al., 2012, Almaghrabi et al., 2014, Adams et al., 2009, Hogaboam and Wallace, 1991), however the effect of CAP and other vanilloid-like agents on platelets from SLE patients, is not currently known.

1.8. Summary and Aims of the Project

This thesis is a continuation of my previous Masters work, and is presented as series of stand-alone manuscripts for publication, and therefore there is a small amount of repetition related to the methods. Previously, I have shown that plant-derived vanilloids, CAP and DHC, and endovanilloids, OLDA and NADA, inhibited *in vitro* ADP-induced platelet aggregation, and CAP, DHC and NADA inhibited AA-induced aggregation (Almaghrabi et al., 2014). Moreover, only endovanilloids showed an effect on aggregation induced by a low concentration (4 μg/mL) of collagen, and thus the collagen pathway was not of interest in the current study (Almaghrabi et al., 2014). Although the effects of vanilloids and endovanilloids on *in vitro* platelet aggregation have been described, the exact mechanism of inhibition of platelet aggregation is unknown. Previous studies investigated the possible mechanism of action of CAP using platelets from healthy individuals, but not other vanilloids or endovanilloids.

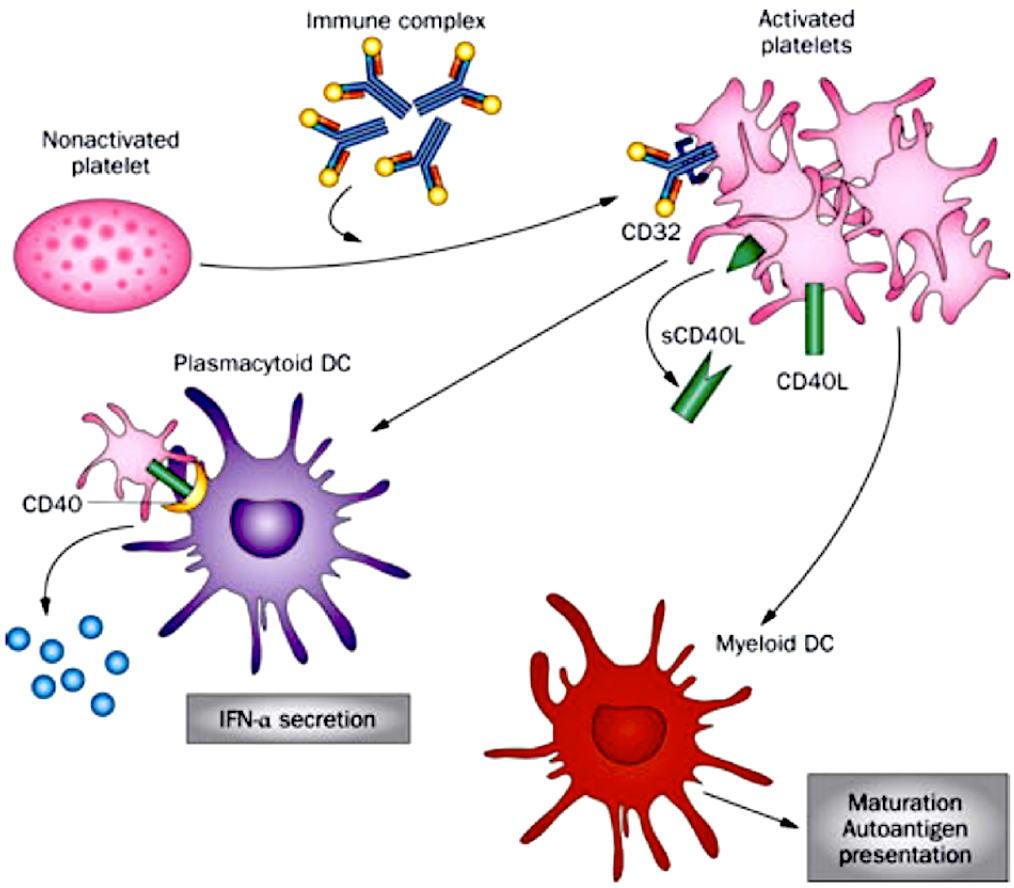

Figure 1-7 Activation of DC and autoantibody production.

Platelets activated by circulating immune complexes express surface CD40L, which promotes platelet aggregation, binding to myeloid DC to induce their maturation and binding to plasmacytoid DC to promote IFN-α secretion. Abbreviations: CD40L, CD40 ligand; DC, dendritic cell; sCD40L, soluble CD40 ligand; SLE, systemic lupus erythematosus. Image sourced from (Boilard et al., 2012) without modifications.

Vanilloid-like agents exert their action in neuronal tissue through TRPV1, CB1 and CB2 receptors, and only a single study has previously shown that TRPV1 is expressed by platelets using Western Blot. Hence, TRPV1 expression by platelets was investigated using, confocal microscopy, and then whether the inhibitory effects of vanilloids are through TRPV1, CB1 and CB2 receptors were determined. Thereafter, whether vanilloids inhibited platelet aggregation was investigated by interfere/block ADP receptors, P_2Y_1 and/or P_2Y_{12}, by measuring granules release, and VASP phosphorylation status, respectively. Furthermore, TXB2 levels were

measured to determine if the inhibitory effect of vanilloids was through suppression of AA pathway. Finally, microparticle release was measured to investigate whether vanilloids suppress platelet aggregation by interfering with their release.

After speculating the mechanism of action of individual vanilloids, it was interesting to know if effect of a combination of CAP and DHC as they occur naturally in *Capsicum* is different compared to the individual vanilloids. Finally, patients who have a high risk of thrombosis and atherosclerosis, SLE patients, were studied as part of a small pilot study to investigate whether vanilloids have any effect, on abnormally hyperactive platelets of SLE patients.

The aims were to:

The overarching aim of this study was to investigate the mechanism of action of plant-derived vanilloids and endovanilloids, and determine the effects of these agents on platelets from SLE patients. The specific aims were to:

1. Determine whether TRPV1 channels are expressed by platelets.
2. Investigate the effects of vanilloids and TRPV1 channels and, cannabinoid CB1 and CB2 receptors antagonists on platelets count, and whether the inhibition of platelet aggregation by vanilloids is mediated through TRPV1 channels and/or CB1 and CB2 receptors.
3. Determine whether vanilloids inhibit *in vitro* platelet aggregation by interfering with one or more of the following: a) the arachidonic acid (AA) metabolic pathway and subsequent thromboxane B2 formation (TXB2); b) adenosine diphosphate (ADP)-induced-dense (5-hydroxytryptamine (5-HT)) release via the P_2Y_1 receptor; c) ADP-induced-α-granules (platelet factor 4

(PF4) and/or β-thromboglobulin (β-TG)) release via the P_2Y_1 receptor; d) vasodilator-stimulated phosphoprotein (VASP) phosphorylation through the P_2Y_{12} receptor.

4. Investigate the effect of vanilloids on platelets microparticles (PMP) formation.
5. Determine the effect of the combination of CAP and DHC on AA-, ADP- and collagen-induced aggregation and TXB2 formation.
6. Investigate the effect of vanilloids on the aggregation of platelets from SLE patients.

2. Chapter 2: The Inhibition of Platelet Aggregation by Vanilloid-like Agents is Not Mediated by Transient Receptor Potential Vanilloid-1 Channels or Cannabinoid Receptors

Part of this chapter has been published as:
Almaghrabi, SY, Geraghty, DP, Ahuja, KDK and Adams, MJ 2016. Inhibition of platelet aggregation by vanilloid-like agents is not mediated by transient receptor potential vanilloid-1 channels or cannabinoid receptors. *Clin Exp Pharmacol Physiol,* 43**,** 606-11.

2.1. Abstract

Vanilloid-like agents, including CAP, N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA) inhibit platelet aggregation, however, little is known about the precise mechanism(s) of action. The aims of the chapter were to; 1) to confirm the presence of transient receptor potential vanilloid-1 (TRPV1) channels in platelets, 2) determine the effect of vanilloid-like agents on *in vitro* platelet count, and 3) investigate whether the effects of vanilloids on collagen-, arachidonic acid (AA)- and ADP-stimulated platelets are mediated by TRPV1 and/or cannabinoid (CB1 and CB2) receptors.

Using confocal microscopy, the presence of TRPV1 was confirmed in platelets. Incubation of platelets with each of the individual vanilloids, CAP, NADA and OLDA, or with TRPV1 (SB452533), CB1 (AM251) and CB2 (AM630) antagonists, for up to two hours did not significantly affect the platelet count. OLDA and NADA significantly inhibited collagen-induced aggregation by 23% and 85%, respectively. SB452533 50 μM and 10 μM significantly (both $p<0.05$) enhanced the anti-aggregating effect of OLDA by 39% and 14%, respectively, compared to OLDA

alone. CAP and NADA significantly inhibited AA-induced aggregation by 56% and 26%, respectively. CAP anti-aggregating effect was enhanced in the presence of SB452533 and AM630 (both 50 μM) by 26.5%, p=0.38 and 30.8%, p=0.19, respectively compared to CAP only, however, the results were not statistically significant. AM251 and AM630 did not affect inhibitory effects of CAP and OLDA on aggregation induced by ADP.

The results suggest that the inhibitory effect of vanilloids on platelet aggregation is independent of TRPV1, CB1 and CB2 receptors. However, blocking these receptors may boost vanilloids anti-aggregating effect. Further research on the antiplatelet activity of vanilloids that focus on mechanisms other than those associated with vanilloid receptors are presented in Chapter 3.

2.2. Introduction

Platelets play a central role in haemostasis and the development of thrombosis by forming adhesive aggregates (Harker et al., 1976, Zucker, 1980). Agents that suppress platelet aggregation are beneficial in the prevention and treatment of cardiovascular diseases (CVD). The active principles of spices such as capsaicin (CAP), from hot chilli peppers, have been reported to affect *in vitro* platelet aggregation (Almaghrabi et al., 2014, Raghavendra and Naidu, 2009, Adams et al., 2009, Harper et al., 2009).

CAP, other capsaicinoids, and endovanilloids including, N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA), act through transient receptor potential vanilloid-1 (TRPV1) in neuronal tissue (Zhong and Wang, 2008), a non-selective cation channel with some preference for Ca^{2+} (Caterina et al., 1999). Endovanilloids

also have affinity for cannabinoid (CB) receptors, and are hence termed endocannabinoids (Hu et al., 2009). TRPV1, CB1 and CB2 receptors are expressed by platelets (Deusch et al., 2004, Catani et al., 2010). One study has shown that TRPV1 channels are present on platelets using Western blotting (Harper et al., 2009), however no information is available on the precise role(s) of these channel on platelet function.

Studies have demonstrated variable effects of CAP on platelet function, including both inhibition (Raghavendra and Naidu, 2009, Adams et al., 2009) and enhancement of aggregation (Harper et al., 2009). Recently, we demonstrated that CAP as well as OLDA and NADA, inhibit ADP-induced *in vitro* platelet aggregation through a TRPV1-independent mechanism (Almaghrabi et al., 2014). OLDA and NADA also suppressed collagen-induced aggregation, while CAP and NADA inhibited aggregation induced by arachidonic acid (AA) (Almaghrabi et al., 2014). Although vanilloid-like agents have been shown to affect platelet aggregation, the mechanism(s) are not well understood, and unlikely to be due to direct toxic effect on platelets (Almaghrabi et al., 2014).

Therefore the aims of this study were to; 1) determine whether TRPV1 channels are expressed by platelets, using confocal microscopy, 2) determine the effects of vanilloids, endovanilloids and receptor antagonists on platelet count, and 3) investigate whether the action of vanilloids/endovanilloids on collagen-, AA- and ADP-induced aggregation is through TRPV1, CB1 and/or CB2 receptors.

2.3. Materials

ADP, AA and collagen were obtained from Helena Laboratories (Beaumont, Texas, USA). CAP, NADA, OLDA; the TRPV1 antagonist SB452533 (N-(2-Bromophenyl)-N'-[2-[ethyl(3-methylphenyl)amino]ethyl]-urea); the CB1 antagonist AM251(N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide); and the CB2 antagonist AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone) were obtained from Tocris (Bristol, UK). ProLong® Gold Antifade Reagent and Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 647 Conjugate) were obtained from Cell Signaling Technology (Boston, MA, USA). Vanilloid receptor 1 (H-150) was obtained from Santa Cruz Biotechnology (Texas, USA). Dako antibody diluent was obtained from Dako (Glostrup, Denmark).

2.4. Methods

Ethics

This study was approved by the Tasmanian Health and Medical Human Research Ethics Committee (approval number: H00011414). Informed written consent was obtained from all participants.

Sample collection and processing for confocal microscopy

Venous blood samples were collected from healthy volunteers (aged 18-65 years),

who had avoided aspirin and antiplatelet medications for at least 10 days and dietary chilli for at least two days. The protocol of isolation of human platelets from whole blood has been used from Abcam® website. Briefly, blood was collected into citrate-phosphate-dextrose buffer (CPD) (16 mM citric acid, 90 mM sodium citrate, 16 mM NaH2PO4, 142 mM dextrose, pH 7.4) as anticoagulant (1:9 ratio of anticoagulant to whole blood). Whole blood was centrifuged at 200g for 20 minutes at room temperature (RT) to obtain PRP. HEP buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 5 mM EGTA, pH 7.4) was added to PRP in 1:1 ratio (v/v), containing prostaglandin E1 (PGE1, 1 μM final concentration) to prevent platelet activation. PRP was centrifuged at 100g for 15 minutes, to pellet red and white blood cells. The supernatant was then transferred into new plastic tubes and centrifuged at 800g for 15–20 minutes to pellet the platelets. The platelet pellet was rinsed with platelet wash buffer (10 mM sodium citrate, 150 mM NaCl, 1 mM EDTA, 1% (w/v) dextrose, pH 7.4) by gently adding wash buffer and removing it slowly with a transfer pipette. The pellet was resuspended in Tyrode's buffer (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1 mM MgCl2, 10 mM HEPES, pH 7.4), containing 5 mM glucose and freshly added BSA (3 mg/mL). The concentration of platelet was adjusted to 1–3 x 10^8 /mL using Tyrode's buffer.

Confocal microscopy

Glass slides were coated with 100 µg/mL fibrinogen in phosphate buffer saline (PBS) overnight at 4°C. They were then blocked with bovine serum albumin (BSA) (5mg/mL) in PBS for 2 hours at RT. Then 1 mL of platelet suspension was added to the slides and allowed to adhere to the blocked slides for 60 minutes at 24°C. The slides were then fixed in 4% paraformaldehyde for 10 minutes at RT, and then washed three times with PBS using a transfer pipette. The slides were incubated with primary antibody (Anti-TRPV1) (1:200 dilution) for 120 minutes, and then a fluorescent secondary antibody (Alexa Fluor 647-conjugated goat anti-rabbit) (1:500 dilution) for 60 minutes. Platelets were mounted using ProLong® Gold Antifade Reagent. Controls included unstained cells to allow for autofluorescence, and secondary antibody only to check for nonspecific fluorescence. These slides were visualized with the laser set at 405 nm to generate bright field images. An UPAL SAPO 60× objective was used and all images were acquired using an Olympus FV1200 Confocal Microscope (Tokyo, Japan) using the same setting and thus comparable.

Sample collection and processing for platelet count and platelet aggregometry

Venous blood was collected by venipuncture using minimal stasis from twenty healthy volunteers (aged 18-65 years) (Table 2-1). Each experiment used platelets from four volunteers. All participants had no previously diagnosed haemostatic abnormality. A total of 20 mL of whole blood was collected into 3.2% sodium citrate anticoagulant tubes (1:9 ratio of anticoagulant to whole blood). Subjects avoided

aspirin and antiplatelet medications for at least 10 days and dietary chilli for at least two days, prior to collection. Samples were centrifuged at 150g for 10 minutes at room temperature to obtain PRP. The remaining blood was then centrifuged at 2000g for 20 minutes at room temperature to obtain platelet poor plasma (PPP).

Table 2-1 participants' characteristics table

Age	Gender	Age	Gender
35	Female	36	Female
32	Female	51	Female
29	Female	35	Female
20	Female	39	Male
24	Female	45	Male
24	Female	24	Male
33	Female	28	Male
32	Female	27	Male
44	Female	43	Male
48	Female	25	Male
25	Female	21	Male
44	Female	24	Male

Platelet count

The effect of vanilloids on *in vitro* platelet count was determined using a Sysmex 1000i analyser (Roche Diagnostics, Sydney, NSW, Australia) with PRP adjusted to 250 x 10^9/L using PPP from the same subject. PRP was used to mimic the same condition of the aggregation experiment (Platelet Aggregometry). CAP, OLDA and

NADA (each 50 and 3.125 μM) and SB452533, AM251 and AM630 (each 50 μM) were added separately to PRP and the platelet count measured every 15 minutes for two hours (n=4, separate subjects). PRP with buffer was used as the blank. Data are presented as percent of platelet count normalised to platelet count at time zero.

Platelet Aggregometry

A four-channel AggRAM platelet aggregometer (Helena Laboratories, Beaumont, Texas, USA) was used to conduct all experiments as described previously (Adams et al., 2009, Ahuja et al., 2009, Ahuja et al., 2012). ADP, AA, collagen, CAP, NADA and OLDA were prepared as previously described (Almaghrabi et al., 2014). Briefly, ADP and AA were dissolved in deionized water and stored in 1M aliquots at -20°C until needed and collagen was ready to use. Aliquots of agonists were thawed and diluted in normal saline (pH 7.1) to produce final concentration of ADP (5 μM), AA (300 μg/mL) and collagen (4 μg/mL). CAP and OLDA were dissolved in 100% ethanol and stored as 0.1M aliquots at -20°C. NADA was stored as 11.37 x 10^3 μM aliquot at -20°C. Aliquots of all vanilloids were diluted as required in normal saline (pH 7.1) to provide final concentrations 50 μM. SB452533 and AM251 were dissolved in 100% ethanol and stored in 10 and 100 mM aliquots, respectively, whereas AM630 was dissolved in DMSO and stored in 100 mM aliquots. All aliquots were stored at -20°C then diluted using normal saline buffer (pH 7.1), to provide final concentrations of 10 and 50 μM for experiments. PPP from the same sample was used as the blank for each experiment and to adjust the platelet count of PRP to 250 x 10^9/L. ADP (5 μM), collagen (4 μg/mL) or AA (300 μg/mL), were added to PRP (225 μL) to initiate aggregation in the presence and absence of TRPV1, CB1 or CB2 antagonists (10, 50 μM), with/out vanilloid (50 μM).

Aggregation was recorded for 10 minutes, with aggregometry data generated by the HemoRAM 1.1.0. Software package (Helena Laboratories, Beaumont, Texas, USA). Parameters included percentage of the maximum aggregation (%MAX) and percentage of the area under the aggregation curve (%AUC). The values for %MAX and %AUC were similar for each experiment, thus only %MAX data (normalised to control, (i.e., agonist only) are presented in figures 2, 3, 5 and 6. Each data set is presented as the mean of four experiments, using platelets from four separate subjects.

Statistical analysis

Data were plotted using GraphPad Prism (version 5; San Diego, CA, USA). All data for comparison between different concentrations of vanilloids with/without receptor antagonists was initially analysed using ANOVA/linear regression (Stata version 13, StataCorp, LP, USA). However, residuals from this model did not conform to the assumptions of linear regression. Therefore, ordinal logistics regression analysis was used for the comparisons, as this model does not require the assumptions of linear regression to be true. Post-estimation Holm test analysis was then used to adjust p values for multiple comparisons. P values < 0.05 were considered statistically significant.

2.5. Results

The presence of TRPV1 channels on platelets

Confocal microscopy demonstrated that TRPV1 channels were present in platelets (Figure 2-1 B,C), with variable expression between, and within, individual platelets. Both secondary antibody (Figure 2-1 D) and unstained cells (Figure 2-1 E) controls did not express TRPV1. The anti-TRPV1 antibody (sc-20813) has been cross-validated by immunodetection in human prostate carcinoma (Czifra et al., 2009), inflamed gingival tissues (Ozturk and Yildiz, 2011) and a synovial joints in a rat model of osteoarthritis pain (Kelly et al., 2015).

The effect of vanilloid-like agents and TRPV1, CB1 and CB2 antagonists on platelet count

Incubation of platelets individually with CAP, OLDA or NADA (each 50 μM), or SB452533, AM251 and AM630 (each 50 μM) for two hours had no statistically significant effect on platelet count, relative to buffer control (Figure 2-2). Similar results were observed with CAP, OLDA and NADA (each 3.125 μM).

Effect of TRPV1, CB1 and CB2 antagonists on collagen-, AA- or ADP-induced aggregation

50 μM of SB452533 (TRPV1 antagonist), AM251 (CB1 antagonist) and AM630 (CB2 antagonist) alone had no significant effect on aggregation induced by collagen, AA or ADP (Figure 2-3). Similar results were observed with 10 μM SB452533, AM251 and AM630. Ethanol (solvent for SB452533 and AM251, as well as the vanilloid-like agents capsaicin, OLDA and NADA), and DMSO (solvent for AM630) also had no effect on platelet aggregation at the final concentrations tested (data not shown).

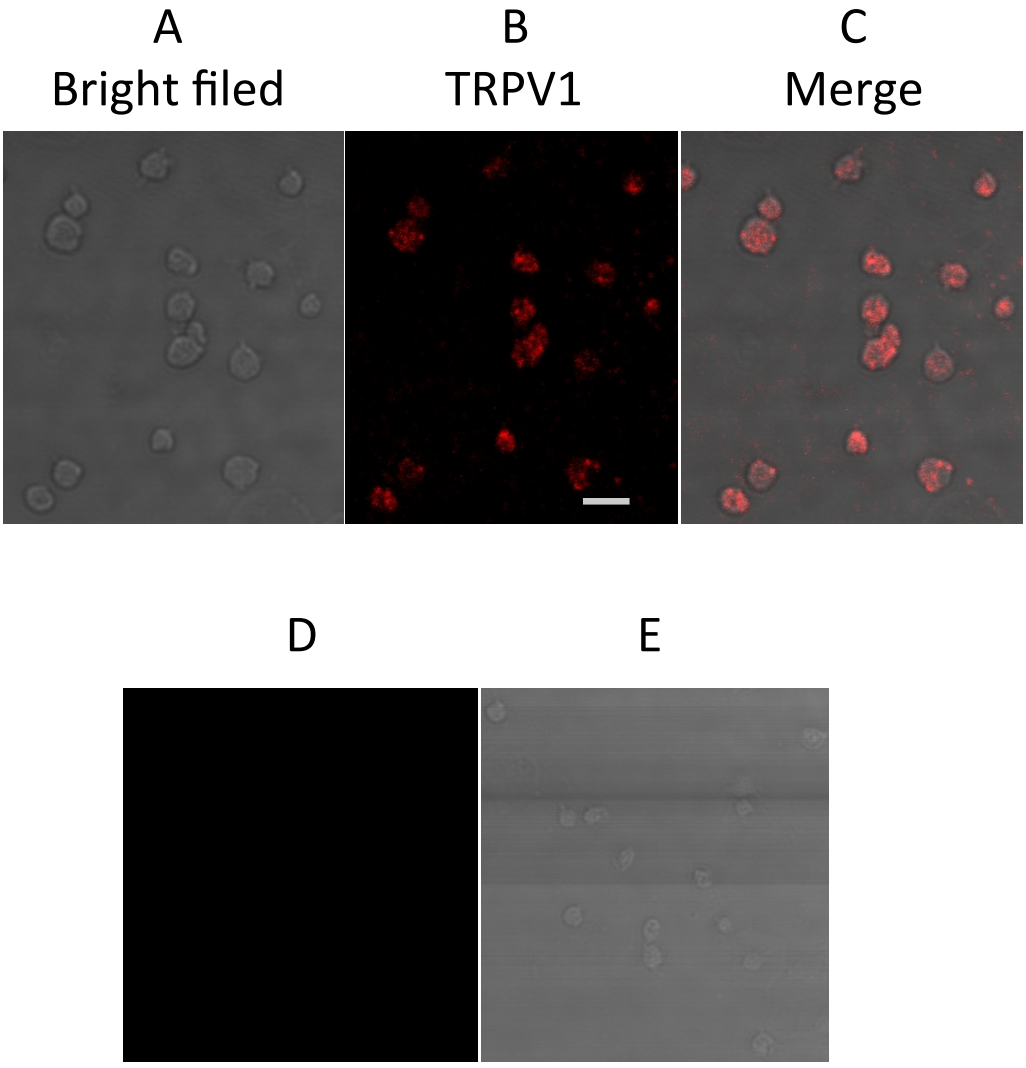

Figure 2-1 Immunodetection of TRPV1 channels on human platelets using confocal microscopy.

All the images are from the same sample, A) Bright field image using 405 nm laser; B) TRPV1 staining of platelets using 647 nm channel; C) merged image of 405 nm and 647 nm channels; D) secondary antibodies only; E) unstained cells. Scale bar= 40 pixels. Original magnification is x 60.

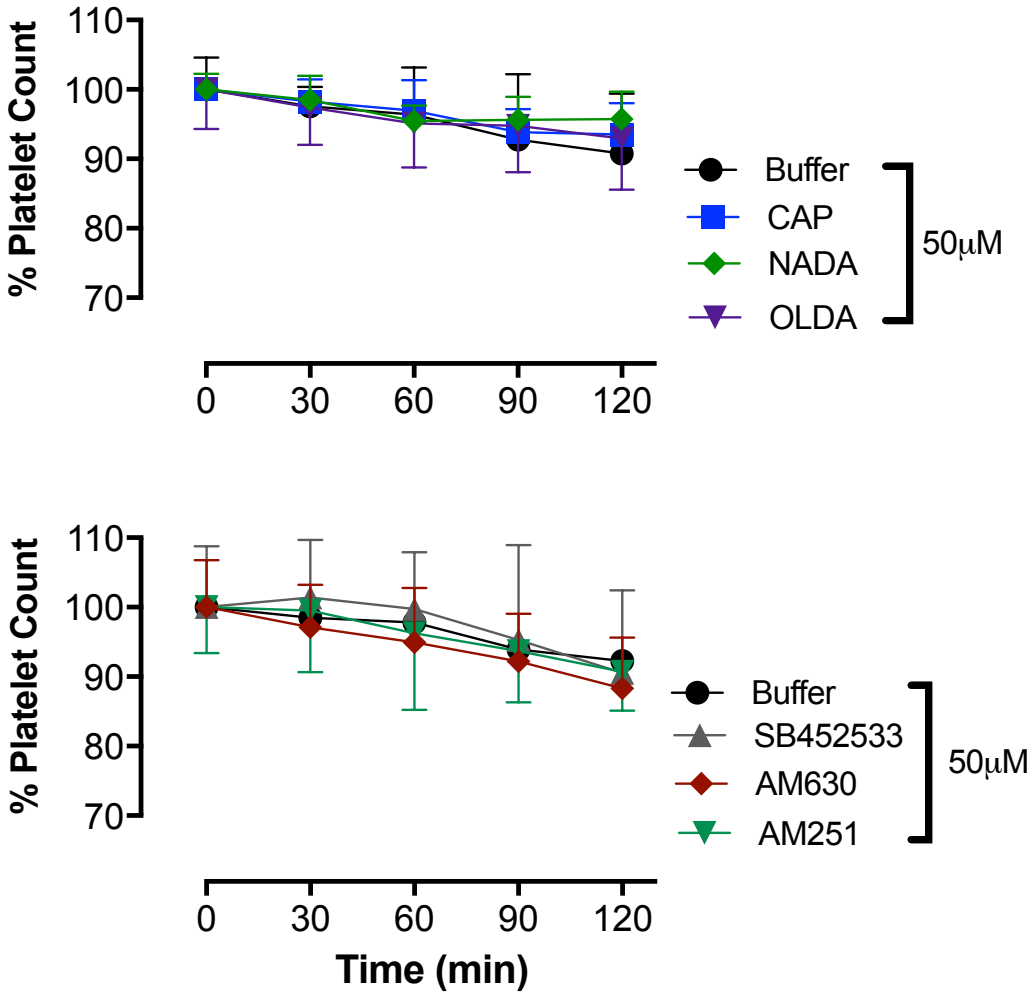

Figure 2-2 50 μM CAP, OLDA, NADA, SB452533 (TRPV1 antagonist), AM251 (CB1 antagonist) and AM630 (CB2 antagonist) had no effect on platelet count over two hours of incubation relative to buffer control.

Data are presented as percent of platelet count (normalised to platelet count at time zero). Mean ± SEM; n=4.

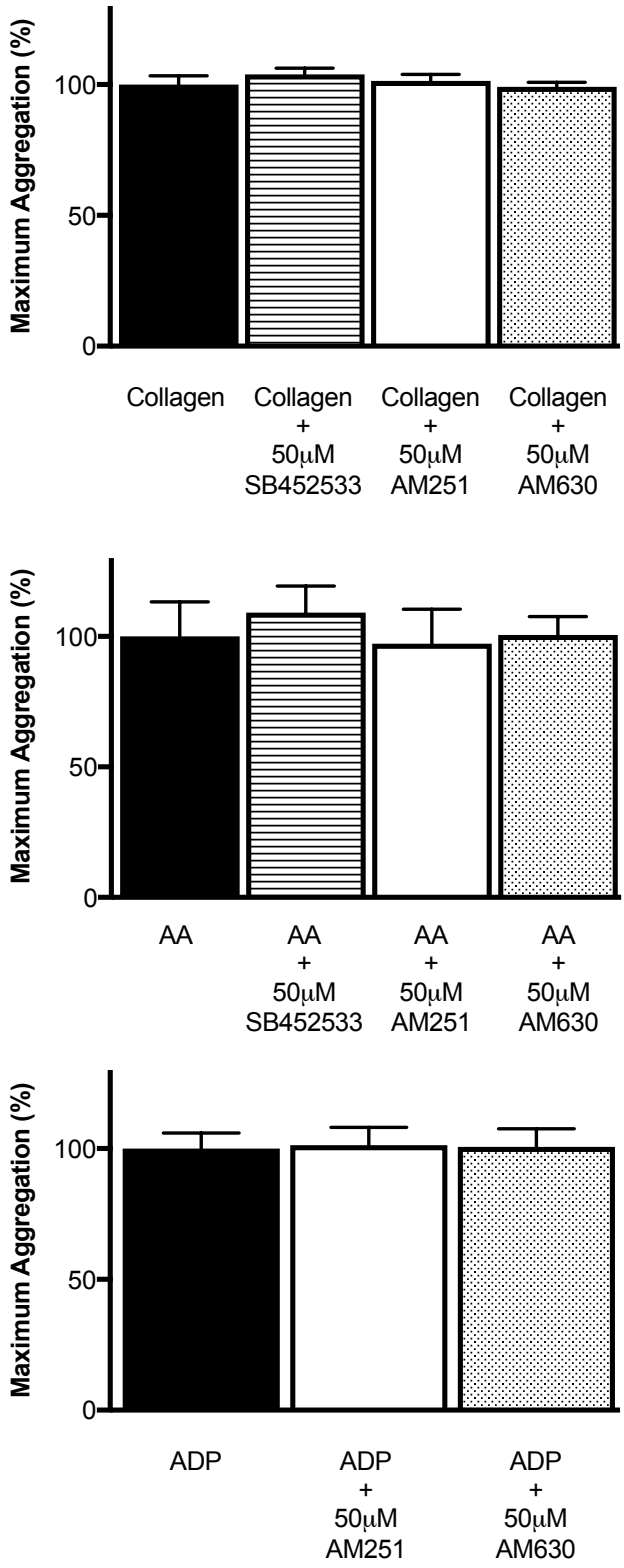

Figure 2-3 Effects of 50 μM SB452533 (TRPV1 antagonist), AM251 (CB1 antagonist) and AM630 (CB2 antagonist) on collagen-, AA- and ADP-induced aggregation.

Data are presented as percent maximum aggregation (normalised to aggregation in the absence of antagonist). Mean ± SEM; n=4.

Effect of blocking TRPV1, CB1 and CB2 receptors on the inhibitory action of OLDA and NADA on collagen-induced platelet aggregation

OLDA and NADA (50 μM) significantly inhibited aggregation induced by collagen by 23%, $p<0.005$ and 85%, $p<0.005$, respectively (Figure 2-4). The inhibitory effect of 50 μM OLDA was not affected by AM251 or AM630 (Figure 2-4 A). However, 50μM SB452533 significantly enhanced the anti-aggregating effect of OLDA (39% compared to the effect of OLDA only, $p<0.05$; Figure 2-4 A and 2-5). A similar effect was observed when 10 μM SB452533 and 50 μM OLDA were combined. The inhibition produced by 50 μM NADA was not affected by SB452533, AM251 or AM630 (10 and 50 μM) (Figure 2-4 B).

Effect of blocking TRPV1, CB1 and CB2 receptors on the inhibitory action of CAP and NADA on AA-induced platelet aggregation

CAP and NADA (50 μM) significantly inhibited AA-induced platelet aggregation by 56%, $p<0.005$ and 26%, $p<0.05$, respectively (Figure 2-6). Moreover, 50 μM of SB452533 and AM630 enhanced the anti-aggregating effect of CAP by 26.5%, $p=0.38$ and 30.8%, $p=0.19$, respectively, compared to CAP only, however the results were not statistically significant. The anti-aggregating effect of CAP in presence of SB452533 (10 μM), AM630 (10 μM), and AM251 (10 and 50 μM) did not change. SB452533, AM251 and AM630 did not have a statistically significant effect on NADA anti-aggregating effect (Figure 2-6).

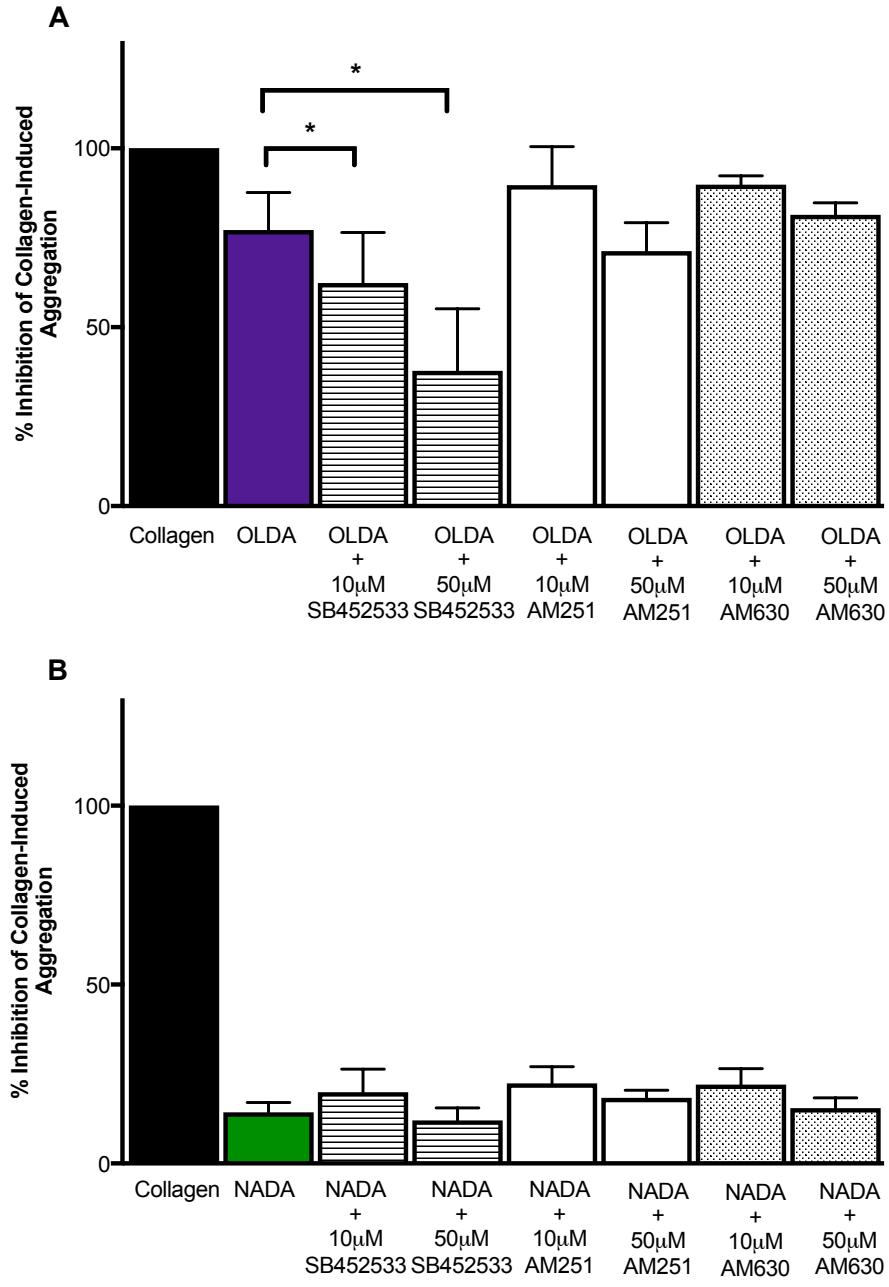

Figure 2-4 Effects of SB452533 (TRPV1 antagonist), AM251 (CB1 antagonist) and AM630 (CB2 antagonist) on (50 μM) OLDA and NADA inhibition of collagen-induced platelet aggregation.

Data are presented as percent maximum aggregation (normalised to aggregation in the absence of vanilloid and antagonist). Mean ± SEM; n=4. *$p<0.05$ compared to control (i.e. 0 μM of antagonist).

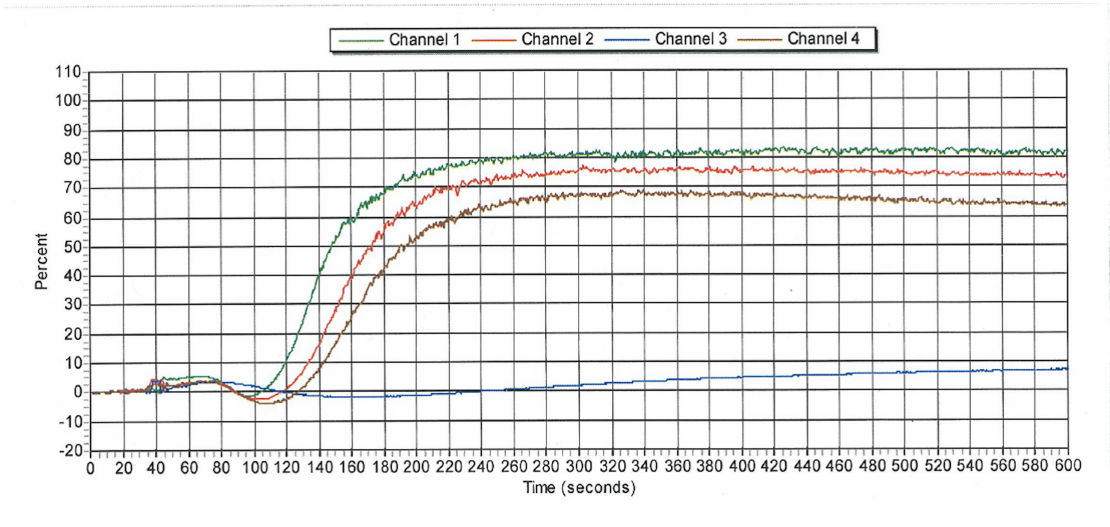

Figure 2-5 Representative aggregation curves (percent aggregation) showing the effect of a TRPV1 antagonist (SB452533) on the inhibitory effect of OLDA on 4µg/mL collagen-induced aggregation.

Green (4µg/mL collagen); brown (4µg/mL collagen + 50 µM OLDA); red (4µg/mL collagen + 50µM SB452533); blue (4µg/mL collagen + 50 µM OLDA + 50µM SB452533) (n=1).

Effect of blocking CB1 and CB2 receptors on the inhibitory action of CAP and OLDA on ADP-induced platelet aggregation

CAP and OLDA (50 µM) significantly inhibited ADP-induced aggregation by 31%, $p<0.05$ and 37%, $p<0.005$, respectively (Figure 2-7). The anti-aggregating effect of CAP and OLDA did not change in the presence of AM251 and AM630 (10 and 50 µM) (Figure 2-7).

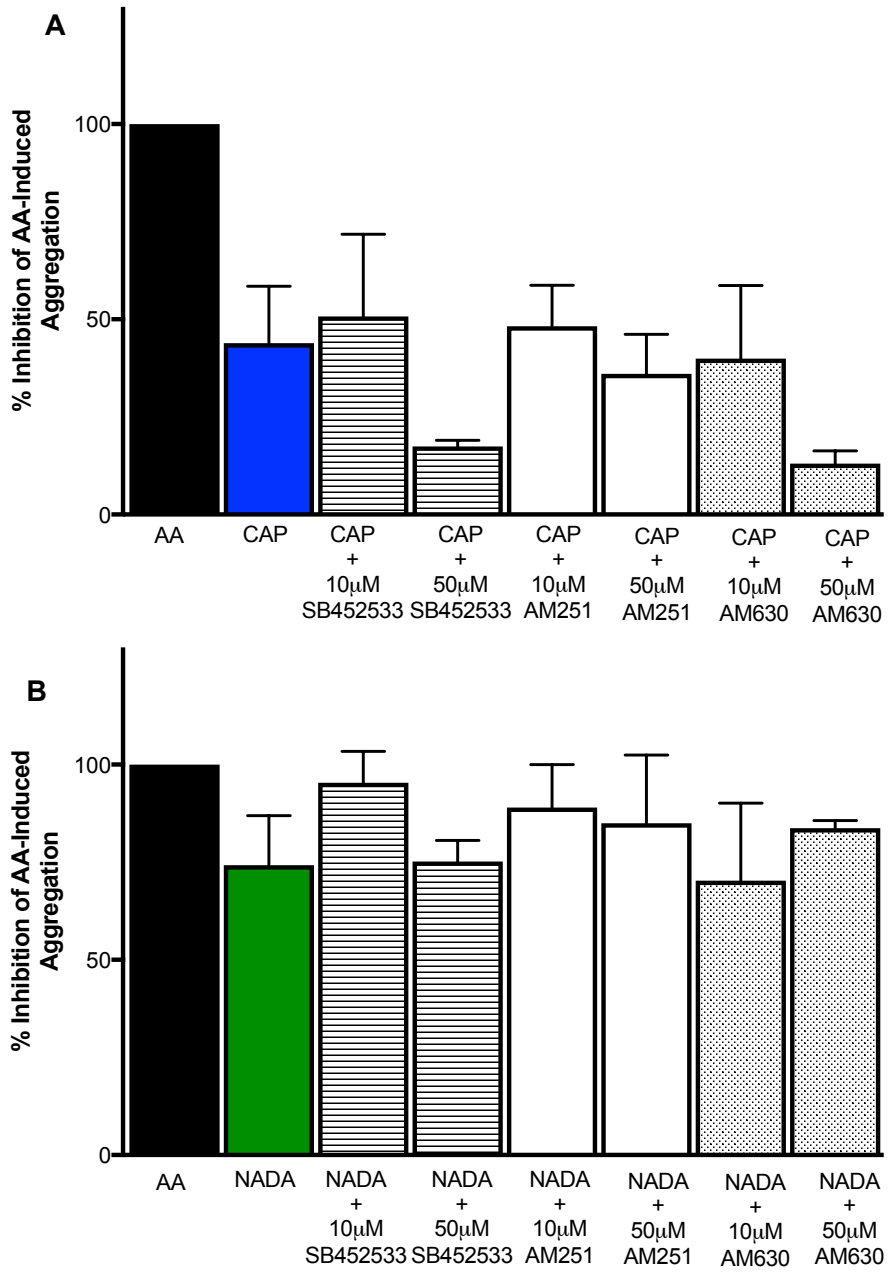

Figure 2-6 Effects of SB452533 (TRPV1 antagonist), AM251 (CB1 antagonist) and AM630 (CB2 antagonist) on (50 μM) CAP and NADA inhibition of AA-induced platelet aggregation.

Data are presented as percent maximum aggregation (normalised to aggregation in the absence of vanilloid and antagonist). Mean ± SEM; n=4.

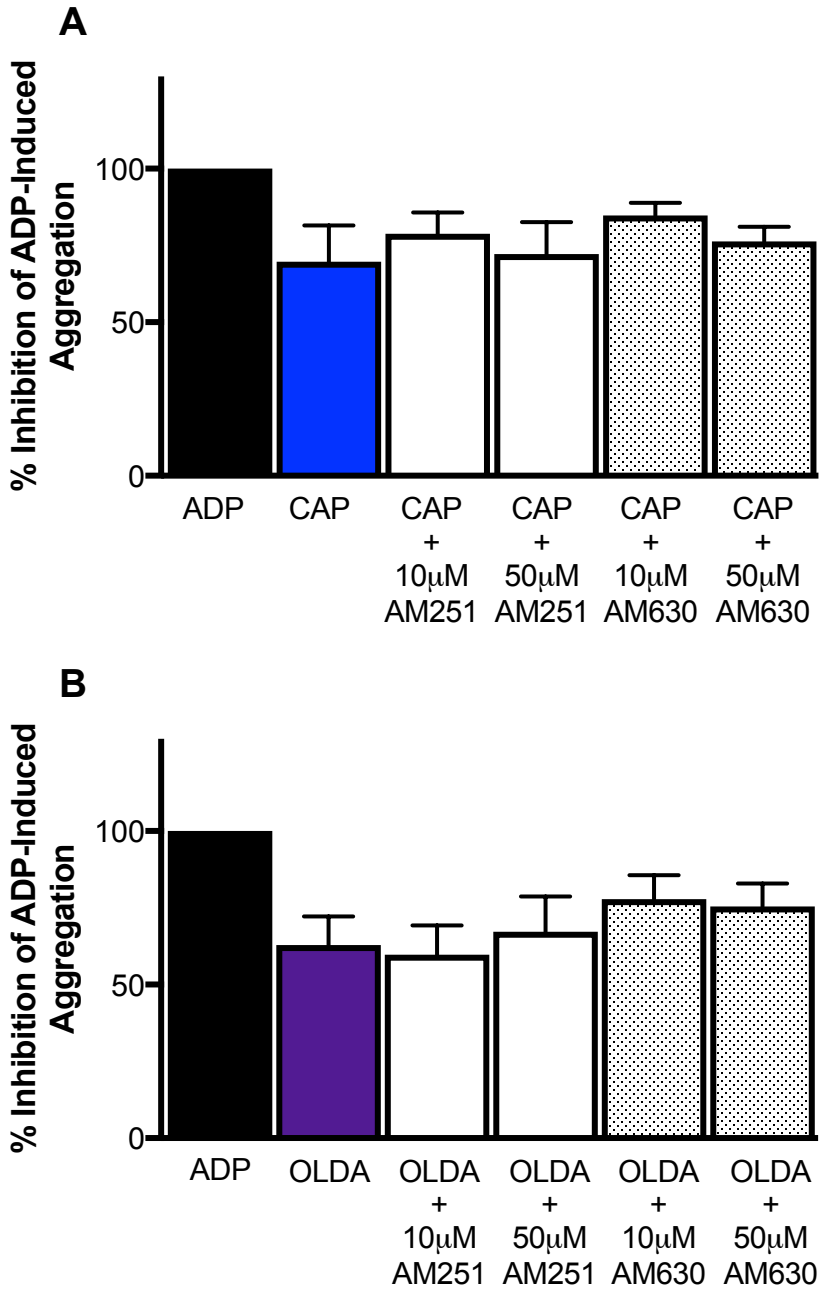

Figure 2-7 Effects of AM251 (CB1 antagonist) and AM630 (CB2 antagonist) on (50 μM) CAP and OLDA inhibition of ADP-induced platelet aggregation.

Data are presented as percent maximum aggregation (normalised to aggregation in the absence of vanilloid and antagonist). Mean ± SEM; n=4.

2.6. Discussion

To the best of my knowledge this is the first study to confirm the presence of TRPV1 channels on platelets using confocal microscopy, and then to investigate the role of TRPV1 channels, and CB1 and CB2 receptors, on the inhibition of platelet aggregation produced by vanilloids and endovanilloids. In this chapter, TRPV1 channels were confirmed to be expressed by platelets, thus its role in the vanilloid inhibition of platelet aggregation could be investigated. Aggregation was induced using three platelet agonists, collagen, AA and ADP, in combination with the presence of vanilloids and/or endovanilloids that previously shown to inhibit aggregation (Almaghrabi et al., 2014). The vanilloids, endovanilloids and receptor antagonists used in this study had no direct effect on platelet count, to rule out the toxic effects of these agents on platelets. Moreover, potent receptors antagonists, SB452533 (TRPV1 antagonist), AM251 (CB1 antagonist) and AM630 (CB2 antagonist) each had no effect on platelet aggregation induced by collagen, AA and ADP, indicating that blocking these receptors individually has no effect on platelet aggregation.

There was no difference in collagen-induced aggregation profile of platelets in presence of NADA or OLDA with/without CB1 and CB2 receptors antagonists. This suggested that the inhibitory effects of OLDA and NADA on aggregation do not appear to be mediated through these receptors. However, blocking TRPV1 channels (with 50 and 10 μM of SB452533) significantly enhanced the inhibitory effect of OLDA but had no effect on NADA. In AA-induced aggregation, CAP in presence of 50 μM TRPV1 and CB2 antagonists showed a greater non-significant inhibitory effect on platelet aggregation compared to CAP alone. Moreover, the inhibitory

influences of NADA on AA-induced aggregation did not appear to be due to a direct interaction with TRPV1, or the CB1 and CB2 receptors. Similar effects were observed with AM251 (CB1 antagonist) and AM630 (CB2 antagonist) on aggregation induced by ADP, as they did not interfere with the anti-aggregating action of CAP and OLDA. Therefore, blocking the TRPV1 channel and CB2 receptor may not impede but enhance vanilloids inhibitory action. However, CB1 receptor appears to have no role in vanilloids effect on platelets.

It has previously been shown that OLDA and NADA inhibited aggregation induced by collagen, while CAP and NADA suppressed AA-induced aggregation (Almaghrabi et al., 2014). Moreover, CAP (Adams et al., 2009, Almaghrabi et al., 2014) as well as the endovanilloid, OLDA, inhibit ADP-induced *in vitro* platelet aggregation, but this effect appeared to be independent of TRPV1 channels (Almaghrabi et al., 2014). Furthermore, the inhibitory effects of vanilloids and endovanilloids did not appear to be mediated by the direct destruction of platelets (Almaghrabi et al., 2014). TRPV1 channels, and CB1 and CB2 receptors, are known to mediate the action of CAP and endovanilloids in several tissues, including neuronal tissue, prostate cells and others (Walker et al., 2005, Van der Aa et al., 2003). However, the role(s) of TRPV1 channels, as well as CB1 and CB2 receptors, expressed by platelets remain unclear (Deusch et al., 2004, Harper et al., 2009, Catani et al., 2010).

In the current study, TRPV1 channels were shown to be present on platelets using confocal microscopy. However, the inhibitory effects of vanilloids and endovanilloids on *in vitro* platelet aggregation, at least in regard to collagen-, AA-

and ADP- induced aggregation of human platelets, are not mediated via TRPV1, nor the CB1 or CB2 receptors. Interestingly, blocking the TRPV1 channels enhanced the inhibitory effect of OLDA in collagen-induced aggregation, and CAP in AA-induced aggregation, which could be due to reduced Ca^{2+} influx $[Ca^{2+}]_i$ and intracellular Ca^{2+} release from platelets and thus greater reduction in Ca^{2+}-mediated aggregation (Clapham, 2003, Montell et al., 2002, Moran et al., 2004). Moreover, CB receptors are coupled through Gi protein and inhibit adenylyl cyclase and cAMP formation (Rueda et al., 2000, Childers and Deadwyler, 1996). Thus, blocking CB2 in presence of CAP in AA-induced aggregation may lead to increase the cAMP formation and further inhibition of platelet aggregation.

Although the exact mechanism for the effects of vanilloids and endovanilloids on non-neuronal tissue remains unclear, CAP has been shown to suppress the AA pathway and thromboxane A_2 formation (Raghavendra and Naidu, 2009) through complete abrogation of AA-induced aggregation (Almaghrabi et al., 2014, Raghavendra and Naidu, 2009). Furthermore, CAP and endovanilloids have been shown to inhibit ADP-induced aggregation (Almaghrabi et al., 2014), an effect postulated to be mediated by the blocking of platelet ADP receptors, and consistent with the demonstration that a two to three fold increase in cAMP completely inhibits ADP-induced aggregation (Haslam et al., 1978). Moreover, it has been demonstrated that CAP is incorporated into phospholipid membranes, resulting in changes to lipid packing and alterations to their thermotropic properties (Aranda et al., 1995). It may be that inhibition of platelet aggregation by vanilloid-like agents could be through incorporation into phospholipid membranes.

It is acknowledged that this study has a number of limitations. Firstly, PRP was used to determine *in vitro* aggregation in response to various agents, which may not accurately reflect *in vivo* platelet function. Secondly, relatively high concentrations of vanilloids/endovanilloids and receptor antagonists were used. However, 50 μM of CAP, OLDA, NADA and the antagonists had no effect on platelet count, and I have previously shown that 50 μM CAP, NADA, OLDA have no effect on platelet viability (Almaghrabi et al., 2014). In rat, 1.24-24.4 percent of the administrated dose of CAP was detected in blood 1-24 hours after oral administration of 30 mg/kg body weight CAP. The maximum concentration of 24.4 percent was seen after one hour and then gradually decreased to 1.24 percent after 24 hours (Suresh and Srinivasan, 2010). Finally, there may be variable responses of platelets from individual subjects related to agonists and/or vanilloids.

In conclusion, the current study demonstrated that platelets express TRPV1 channels and vanilloids/endovanilloids and their respective antagonists have no effect on platelet count. Blocking TRPV1 channels, and CB1 and CB2 receptors alone had no effect on platelet aggregation. However, blocking TRPV1 and CB2 receptor in a combination with OLDA and CAP appears to enhance vanilloids inhibitory effect. Thus further investigation in these receptors role/s in platelets is warranted. Platelets play an important role in contributing to the formation of atherosclerotic lesions, a major contributing factor to the development of CVD. Naturally occurring antiplatelet compounds such as vanilloid-like agents may potentially exhibit fewer adverse effects, e.g., gastrointestinal disturbances, than existing medications and may be useful as an adjuvant or alternative to conventional antiplatelet medications.

Further research is warranted to determine the precise mechanism(s) of action of vanilloids/endovanilloids and their suitability for therapeutic application.

3. Chapter 3: Inhibition of Vanilloid-Induced in vitro Platelet Aggregation is Dependent on VASP Phosphorylation and Thromboxane Biosynthesis but Independent of Platelet Granule Release

3.1. Abstract

The mechanism by which vanilloids, including plant-derived vanilloids (capsaicin (CAP) and dihydrocapsaicin (DHC)), and endogenous vanilloids (N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA)), inhibit platelet aggregation is not known. The aims of this study were to determine whether vanilloids inhibit *in vitro* platelet aggregation by interfering with one or more of the following: 1) vasodilator-stimulated phosphoprotein (VASP) phosphorylation, 2) adenosine diphosphate (ADP)-induced dense (5-hydroxytryptamine (5-HT)) release, 3) α-granules (platelet factor 4 (PF4) and β-thromboglobulin (β-TG)) release, and 4) the arachidonic acid (AA) metabolic pathway and subsequent thromboxane B2 formation (TXB2). In addition, the effect of vanilloids on platelet-derived microparticles (PMP) formation/release was investigated.

Platelets were obtained from venous blood of healthy volunteers. VASP phosphorylation was measured (ELISA) after incubation of platelets with 3.125 and 50 μM CAP, DHC, NADA and OLDA. 5-HT, PF4 and β-TG release from dense and α-granules were measured (ELISA), in the presence and absence of 3.125, 25 and 100 μM of each of the vanilloids on platelets treated with 10 and 5 μM ADP. Platelets were treated with AA (300 μg/mL) in the absence and presence of 3.125 and 50 μM CAP, DHC, NADA and OLDA, TXB2 formation determined (ELISA).

PMP release was measured in the presence and absence of vanilloids (50 μM) in ADP- and AA-activated platelets by flow cytometry.

NADA increased VASP phosphorylation significantly (17% ± 2.2, $p<0.05$) compared to control (no treatment control), and OLDA had a non-significant effect on VASP phosphorylation (13.4% ± 2.7, $p=0.12$). Vanilloids did not significantly affect the release of PF4, β-TG or 5-HT from ADP-activated platelets. CAP (50 μM) significantly inhibited TXB2 formation from AA-activated platelets (10.7%, $p<0.001$) and to a lesser extent DHC (50 μM; 4.6%; $p=0.91$). None of the vanilloids had a statistically significant effect on PMP release from ADP- and AA-stimulated platelets.

The results of this study suggest that the inhibitory effect of NADA on *in vitro* platelet aggregation might be through interference with the ADP receptor, P_2Y_{12}, as VASP phosphorylation increased significantly in its presence. Furthermore, CAP may inhibit platelet aggregation by suppressing the AA metabolic pathway. The inhibitory effects of CAP, DHC, OLDA and NADA on *in vitro* platelet aggregation do not appear to be mediated through inhibition of the P_2Y_1 ADP receptor, as dense- and α-granules release were not affected. Finally, the inhibition of aggregation by vanilloids is not related to a reduction in PMP formation/release.

3.2. Introduction

Platelets are small anucleate cell fragments that originate from megakaryocyte cytoplasm and play a crucial role in the hemostatic process and in thrombus formation (Zucker, 1980). There are many endogenous agonists that activate platelets and act through various signaling pathways. *In vivo*, thrombin and collagen initiate

platelet activation, however several other agonists can also stimulate platelets *in vitro*, including adenosine diphosphate (ADP) and arachidonic acid (AA) (Del Conde et al., 2005, Falati et al., 2003).

The activation of platelets through collagen glycoprotein receptors ($\alpha_2\beta_1$ and GPVI) leads to the release of thromboxane A2 (TXA2) and ADP (Clemetson et al., 1999, Maffrand et al., 1988) via phospholipase C (PLC) activation. As a result of PLC activation, the cytosolic Ca^{2+} concentration increases leading to activation of phosphokinase C (PKC) and then phospholipase A (PLA) (Lemons et al., 2000, Jurk and Kehrel, 2005). PLA then mediates AA release from phospholipid membranes, cyclooxygenase-1 (COX-1) converts AA into prostaglandin H2 (PGH2), and the latter converts to TXA2 via TXA2 synthase (Stassen et al., 2004, Patrono, 1994). However, TXA2 has a short half-life and undergoes non-enzymatic hydration to form thromboxane B2 (TXB2), which is more stable and measurable (Catella et al., 1986).

Platelets express two ADP receptors, P_2Y_1 and P_2Y_{12}, which belong to the purinergic class of G-protein-coupled receptors (GPCRs) (Daniel et al., 1998, Jantzen et al., 1999). Activation of P_2Y_1 enhances the cytosolic Ca^{2+} concentration that leads to the release of the contents of α- and dense-granules (Daniel et al., 1998, Siess, 1989). ADP binds to the P_2Y_{12} receptor on the platelet surface and signals via G_i inhibitory protein to inhibit adenyl cyclase, cAMP formation and dephosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Cattaneo, 2007). VASP is an intracellular platelet protein, which is unphosphorylated at the basal state and plays a significant role in negatively regulating adhesive events that are involved in platelet

aggregation (Halbrugge et al., 1990, Waldmann et al., 1987, Aszodi et al., 1999).

Platelet derived microparticles (PMP), which are shed fragments of the plasma membrane of platelets, are the most abundant microparticles (MP) in the circulation (~70% to 90% of total) (Horstman and Ahn, 1999, Joop et al., 2001, Berckmans et al., 2001). PMP are typically characterised by size (<1 μm), as well as expression of either GPIb or $\alpha_{IIb}\beta_3$ receptors (Flaumenhaft et al., 2009). In general, MP consist of a phospholipid bilayer, which encloses transmembrane receptors and proteins, and exposes intracellular components including transcription factors, enzymes and mRNA derived from MPs parent cells (VanWijk et al., 2003). The circulating level of PMP is a marker of platelet activation (Abrams et al., 1990, Morel et al., 2011a, Keuren et al., 2006). PMP have important physiological roles, including as signaling structures between cells (Morel et al., 2004). In addition, PMP participate in the pathogenesis of atherosclerosis and thrombus formation, by providing a catalytic phospholipid surface for the assembly of blood coagulation factors. Furthermore, they expose phosphatidylserine and express tissue factor, thereby promoting thrombin generation and amplification of coagulation (Tan and Lip, 2005, Lee et al., 1993, Mallat et al., 2000, Lacroix et al., 2013). The significant roles of PMP in the development of thrombosis and atherosclerosis make them a potentially attractive therapeutic target for the treatment and/or prevention of cardiovascular diseases (CVD) (Abrams et al., 1990, Morel et al., 2011a, Keuren et al., 2006, Azevedo et al., 2007, Hartopo et al., 2016). To date, nothing is known about the effect of vanilloids on these biomarkers (i.e., PMP) of thrombosis.

Agents that suppress platelet activation and aggregation are well known for their

importance in preventing and treating CVD. Natural compounds such as the active ingredient of the hot chilli pepper have been studied as potential antiplatelet agents (Adams et al., 2009, Almaghrabi et al., 2014, Mittelstadt et al., 2012, Raghavendra and Naidu, 2009, Hogaboam and Wallace, 1991). Hot chilli peppers contain capsaicinoids, a group of compounds that give them their characteristic pungent taste. The two major capsaicinoids responsible for pepper pungency are capsaicin (CAP) and dihydrocapsaicin (DHC) (Kozukue et al., 2005, Curry et al., 1999). CAP, DHC and endogenous vanilloid-like lipids ('endovanilloids'), e.g., N-oleoyldopamine (OLDA) and N-arachidonoyldopamine (NADA), inhibit *in vitro* ADP- and AA-induced platelet aggregation (Adams et al., 2009, Almaghrabi et al., 2014, Raghavendra and Naidu, 2009). In addition, CAP and endovanilloids exert their action in sensory neurons by activating transient receptor potential vanilloid-1 (TRPV1) and cannabinoid receptors (CB) (Caterina et al., 1999, Hu et al., 2009). TRPV1 and CB receptors are present in/on platelets but neither CAP nor endovanilloids act through these receptors to inhibit platelet aggregation (Harper et al., 2009, Deusch et al., 2004, Mittelstadt et al., 2012), as I have earlier shown in Chapter 2. Therefore, the precise mechanism/s of action of vanilloids and endovanilloids on platelet aggregation remain to be elucidated.

In this study, whether vanilloids inhibit *in vitro* platelet aggregation by interfering with one or more of the following was investigated: 1) ADP-induced VASP dephosphorylation, 2) ADP-induced dense (5-hydroxytryptamine (5-HT)) release, 3) ADP-induced α-granules (platelet factor 4 (PF4) and β-thromboglobulin (β-TG)) release, 4) the AA metabolic pathway and subsequent TXB2 formation. Furthermore, the effects of vanilloids on the formation/release of PMP were also determined.

3.3. Materials

ADP and AA were purchased from Helena Laboratories (Beaumont, Texas, USA). CAP, NADA and OLDA were obtained from Tocris (Bristol, UK). DHC, 1.1μm and 0.8μm carboxylate and amine modified latex (polystyrene) beads, were obtained from Sigma-Aldrich (St Louis, USA). The following ELISA kits were used: TXB2 (Abcam, Melbourne, Australia), 5-HT, PF4 and β-TG (Uscn Life Science Inc, Wuhan, China), VASP (Diagnostica Stago, Melbourne, Australia). Hank's Balanced Salt Solution (HBSS) was obtained from Thermo Fisher Scientific (Victoria, Australia). BV421 anti-human CD41b and PE anti-human CD42a antibodies were bought from BD Biosciences (Victoria, Australia).

3.4. Methods

Ethics

This study was approved by the Tasmanian Health and Medical Human Research Ethics Committee (approval number: H00011414). Informed written consent was obtained from all participants.

Sample collection

Venous blood was collected by venipuncture using minimal stasis from healthy volunteers (aged 18-65 years). All participants had no previously diagnosed haemostatic abnormality. Subjects avoided aspirin and antiplatelet medications for at least 10 days and dietary chilli for at least two days, prior to collection.

Vanilloids and platelets agonist preparation

CAP, DHC, OLDA and NADA, and platelet agonists, ADP and AA, were prepared as previously described in Chapter 2.

Effects of vanilloids and endovanilloids on VASP phosphorylation status

VASP dephosphorylation/phosphorylation status was determined using ELISA in the absence and presence of 3.125 and 50 μM of CAP, DHC, NADA and OLDA according to manufacturer's instructions (Appendix 3). The concentration of ADP used in these kits is unknown and when I contacted the company they declined to disclose such information. The blood was incubated with the reagents for 10 minutes. The measurement of VASP phosphorylation, expressed as platelet reactivity index (PRI), mirrors the degree of P_2Y_{12} receptor inhibition, i.e., the lower the PRI, the higher the inhibition of the P_2Y_{12} receptor. The PRI was calculated using optical density (OD) at 450 nm in the presence of prostaglandin E1 alone [PGE1], or PGE1 and ADP simultaneously [PGE1+ ADP], using this formula:

PRI% = (OD[PGE1] – OD[PGE1+ADP]/ OD[PGE1] – OD(blan)) x100.

Effects of vanilloids and endovanilloids on 5-HT, PF4 and β-TG release from platelets

The release of dense and α-granules from platelets treated with 5 and 10 μM ADP in the absence and presence of 3.125, 25 and 100 μM of CAP, DHC, NADA and OLDA was measured using ELISA kits for 5-HT, PF4 and β-TG, according to manufacturer's instructions (Appendix 4-6). Vanilloids were incubated with platelets

for 10 minutes. Data are presented as percent 5-HT, PF4 and β–TG normalised to their concentration with ADP only (i.e. no treatment control).

Effects of vanilloids and endovanilloids on TXB2 release from platelets

TXB2 is a stable metabolite of TXA2 (Catella et al., 1986). The measurement of TXB2 release from platelets treated with AA (300 μg/mL) in the absence and presence of 3.125 and 50 μM of CAP, DHC, NADA and OLDA was performed using a TXB2 ELISA kit according to manufacturer's instructions (Appendix 7). Platelets were incubated for 10 minutes with vanilloids. Data are presented as percent TXB2 concentration normalised to TXB2 concentration in plasma with AA only (i.e. no treatment control).

Effects of vanilloids and endovanilloids on the release of Platelet-derived microparticles

Preparation of microparticles

Venous whole blood was collected by venipuncture from four volunteers (aged 18-65) with 21-gauge needles. Samples were collected into acid citrate dextrose anticoagulant (ratio blood:anticoagulant 9:1) and processed within two hours. All experiments were conducted as previously described (van der Heyde et al., 2011). Briefly, blood was centrifuged at 150g for 10 minutes to generate platelet rich plasma (PRP). Platelets were purified by washing PRP using Ca^{2+} and Mg^{2+} free Tyrode's buffer (140mM NaCl, 3 mM KCl, 47 mM NaH2PO4, 1.2mM NaHCO3, 1 mg mL^{-1} glucose, pH 7.4). Tyrode's buffer was sterile-filtered with 0.2 μm filter, to

reduce the background noise (Dey-Hazra et al., 2010). The platelet count was determined using a Sysmex 1000i analyser (Roche Diagnostics, Sydney, Australia) and adjusted to 1x 10^6/mL using Tyrode's buffer. Platelets were then activated and incubated for 15 minutes with ADP (5 μM) or AA (300 μg/mL) in the presence and absence of CAP, DHC, NADA or OLDA (final concentrations 50 μM). Samples were then centrifuged at 5000g for 15 minutes, and the cell-free supernatant rich in MP, including PMP, transferred to a separate tube.

Flow cytometry

The MP rich supernatant (10 μL) was added into flow cytometry tubes, labeled with 5 μL of CD41b-BV421 and CD42a-PE and incubated in the dark for 25 minutes. Subsequently, 500 μL of Hank's Balanced Salt Solution (HBSS) was added then the samples subjected to flow cytometry. Data were acquired using an Attune® Acoustic Focusing Cytometer (Life Technologies, Grand Island, USA). Fluorescence and scatter signals were calibrated with Attune™ Performance Tracking Beads (Thermo Fisher Scientific, Victoria, Australia). Latex beads, 1.1 and 0.8 μm, were run and used to set the upper limit of the population to analyse with FSC <1.0 μm (Figure 3-1 a). PMP were gated by their size (<1.0 μm) and positivity for anti-CD41b and -CD42a (Figure 3-1 b,c). For each sample there were eight controls including, PRP, MP plasma only, MP + ADP, MP + AA, MP + CAP, MP + DHC, MP + OLDA and MP + NADA, as the biological comparison controls are the most appropriate to set positive/negative limits (Maecker and Trotter, 2006).

Statistical analysis

One-way ANOVA was performed using GraphPad Prism. Holm-Šidák was used to

adjust p-values for multiple comparisons. $P < 0.05$ was considered statistically significant (version 7; San Diego, CA, USA). Analysis of the flow data was performed using dot plot that was collected from fluorescence data acquired in the logarithmic mode from 10,000 events analysed in each sample. Final analysis was performed using FlowJo (version 7/9, Oregon, U.S.A.). Data were plotted using GraphPad Prism (version 7; San Diego, CA, USA) and expressed as the percentage of fluorescence-positive MP and normalised to MP plasma only.

3.5. Results

The effect of vanilloids and endovanilloids on VASP phosphorylation

NADA (50 μM) significantly reduced the PRI (increased VASP phosphorylation) by 17% ± 2.2, $p<0.05$ compared to control (no treatment control) (control PRI = 98.9%), whereas OLDA decreased the PRI, but this was not statistically significant (13%± 2.7, $p=0.12$). CAP and DHC had minimal effect on VASP phosphorylation (Figure 3-2).

The effect of vanilloids and endovanilloids on the release of 5-HT, PF4 and β-TG from platelets

Relative to ADP alone, neither plant-derived vanilloids (CAP and DHC), nor endogenous vanilloids (NADA and OLDA) (3.125, 25 and 100 μM) had a significant effect on 5-HT, PF4 or β-TG release on ADP-activated platelets (Figure 3-3).

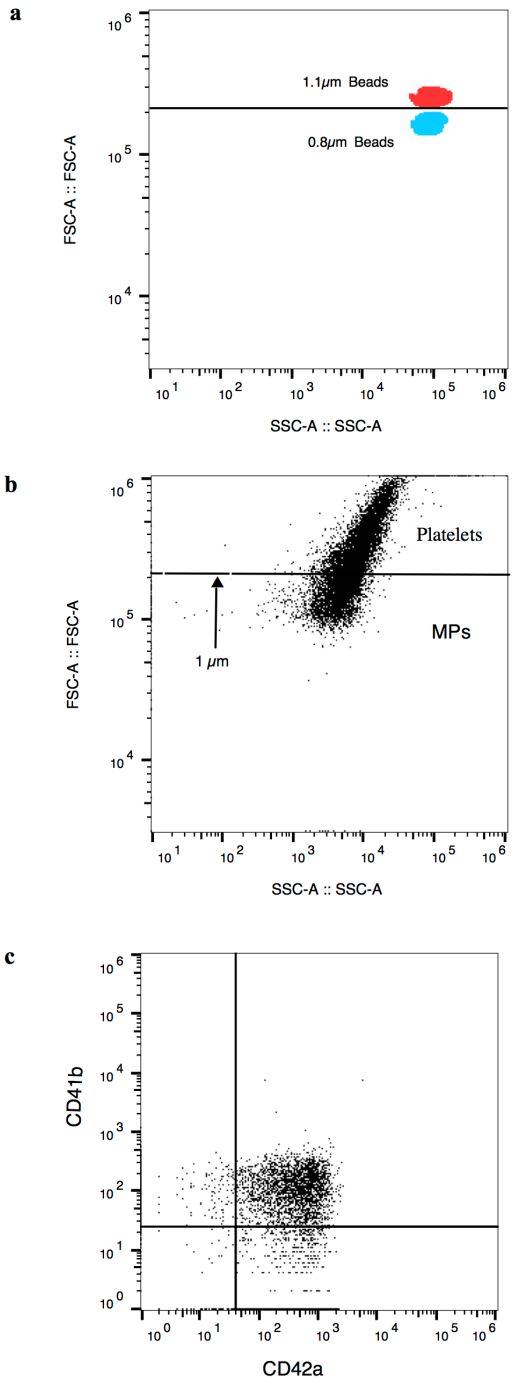

Figure 3-1 Flow cytometric analysis and quantification of MP and PMP.

(a) Forward and side scatter gating showing the 0.8 and 1.1 μm beads and the upper limit of the MP gate. (b) Dot plots graph of plasma that has not been completely depleted in platelets, MP and platelets can be easily distinguished using 1 μm limit. (b) Double staining of MP plasma using CD41a and CD42b.

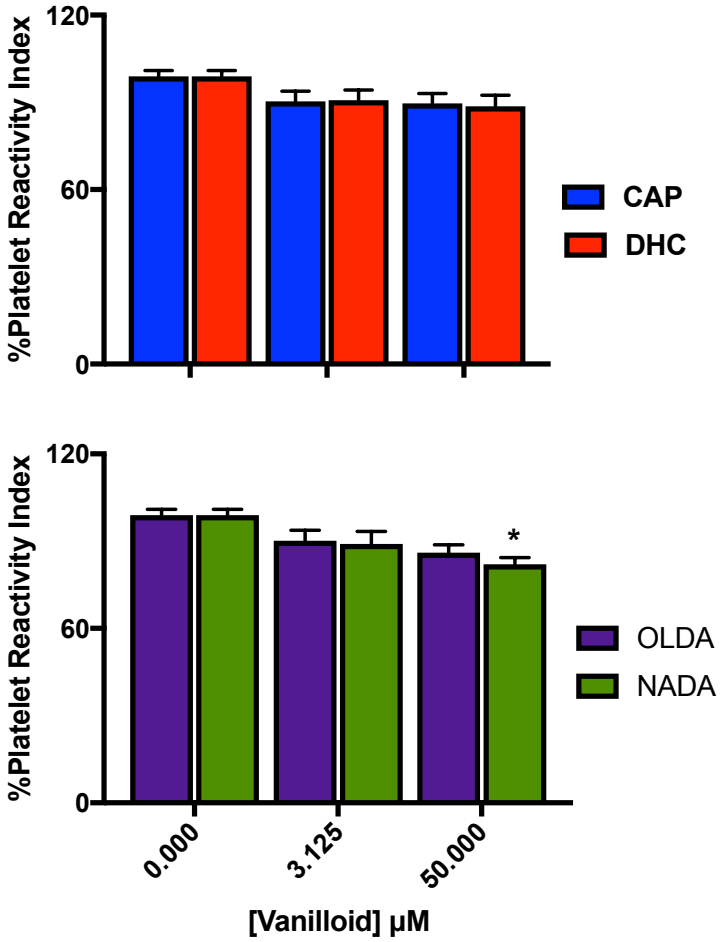

Figure 3-2 Effect of CAP, DHC, NADA and OLDA on VASP phosphorylation status.

Data are presented as percent of platelet reactivity index (PRI), n=6, * $p < 0.05$.

The effect of vanilloids and endovanilloids on the release of TXB2 from platelets

TXB2 release was decreased significantly in the presence of CAP (50 µM; 10.7%, $p<0.001$) following stimulation with AA (Figure 3-4). DHC (50 µM; 4.6%) decreased TXB2 release but this result was not statistically significant ($p=0.80$). However, 3.125 µM CAP and DHC had no effect on TXB2 formation. Furthermore,

neither OLDA nor NADA (3.125 and 50 μM) had an effect on TXB2 compared to AA alone (no treatment control) (Figure 3-4).

The effect of vanilloids and endovanilloids on the release of platelet microparticles

A gate was set to include only events showing FSC <1 μm beads. If the MP were stained, then another gate was set to include only events with fluorescence intensity higher than that detected with unstained MP.

In comparison to MP only plasma, ADP stimulated a slight increase in MP generation from platelets in the presence and absence of vanilloids (Figure 3-5A and 3-6A). The quantities of CD41b and CD42a positive PMP (indicative of glycoprotein GPIIb and integrin alpha IIb, and GPIX, respectively) remained unchanged after exposure to ADP (5 μM). Although CAP, DHC, OLDA and NADA (50 μM) alone decreased the number of CD41b and CD42a positive PMP (~24%), the decreases were not statistically significant (Figure 3-5B and 3-6B).

In comparison to ADP (5 μM) alone, plant-derived vanilloids and endovanilloids had no effect on MP generation by ADP-stimulated platelets (Figure 3-5A and 3-6A). Moreover, CAP did not change the generation of CD41b/CD42a positive PMP whereas DHC slightly increased it (Figure 3-5B). NADA non-significantly increased the CD41b and CD42a positive PMP by 17.2% (p=0.91) while OLDA had no effect (Figure 3-6B).

In comparison to MP only plasma, AA (300 μg/mL) stimulated a slight increase in MP generation in the absence and presence of CAP and DHC (Figure 3-7A), but none were statistically significant. Generation of PMP (CD41b/CD42a positive) was reduced (~25%) by AA, CAP and DHC alone (Figure 3-7B). Interestingly, PMP generation increased in the presence of AA+CAP and AA+DHC by 37.3% (p=0.24) and 11% (p=0.99), respectively, compared to AA only (Figure 3-7B).

A similar pattern was observed for NADA and OLDA, i.e., slight increases in MP generation with AA or vanilloids alone (Figure 3-8A), but there was a non-significant reduction in PMP generation by NADA and OLDA alone, and in combination with AA (Figure 3-8B). Results obtained in these experiments are summarised in Table 3-1.

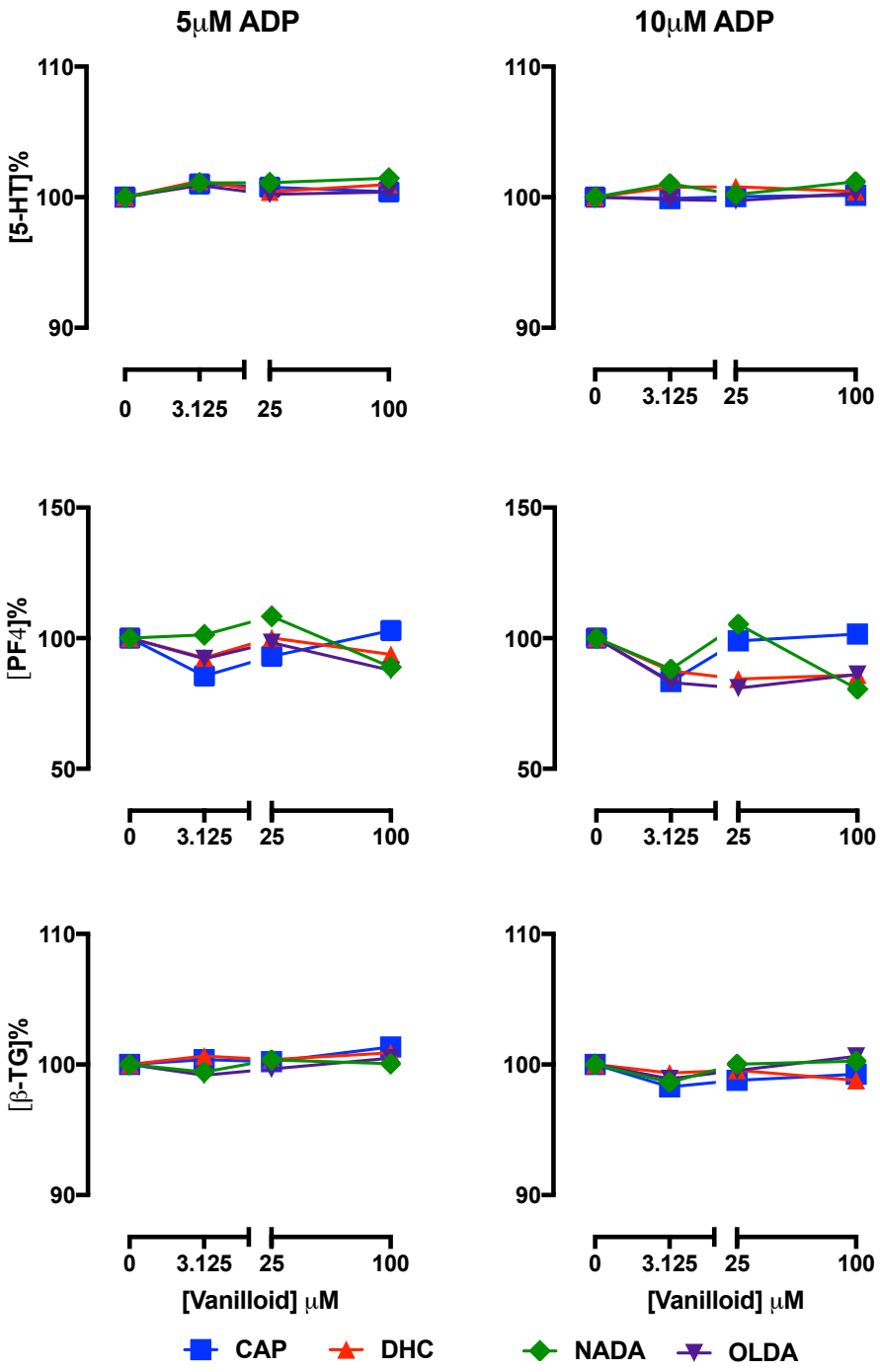

Figure 3-3 Effect of CAP, DHC, NADA and OLDA on 5-HT, PF4 and β–TG release.

Data are presented as percent of 5-HT, PF4 and β–TG concentration normalised to their concentration with ADP only (i.e. no treatment control). Results are the mean ± SEM, n=4. Some error bars are too small to be seen.

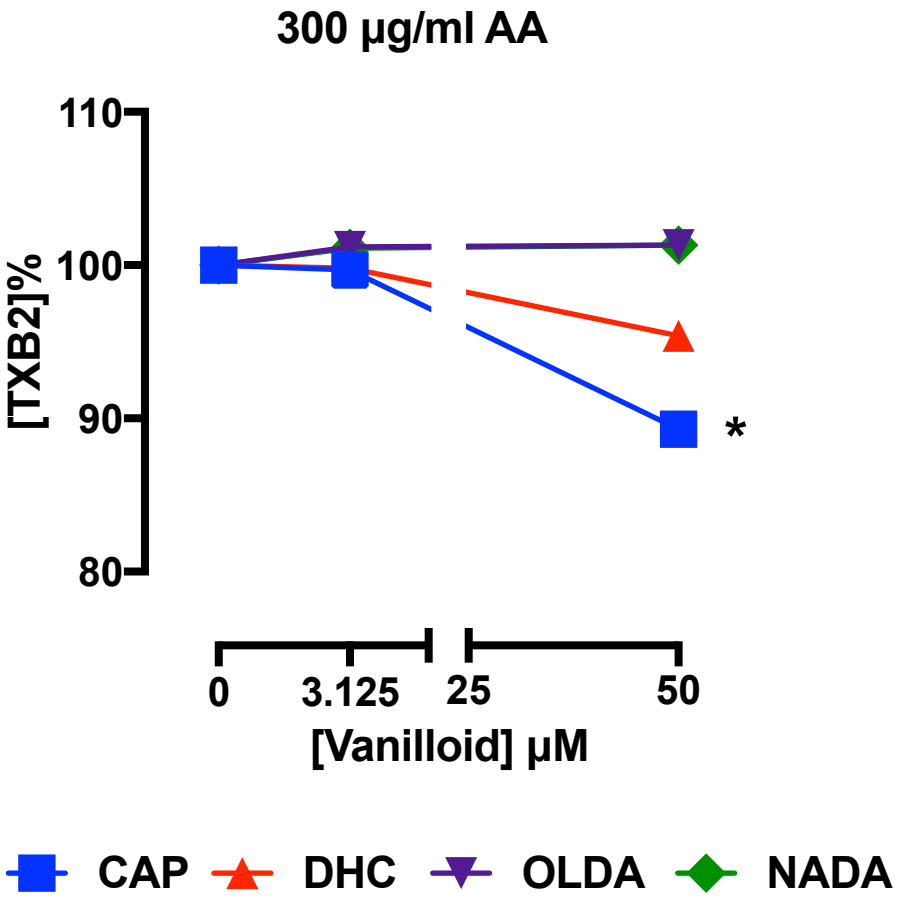

Figure 3-4 Effect of CAP, DHC, NADA and OLDA on TXB2 formation.

Data are presented as percent of TXB2 concentration normalised to TXB2 concentration with AA only (i.e. no treatment control). Results are the mean ± SEM, n=5, *p<0.001. Some error bars are too small to be seen.

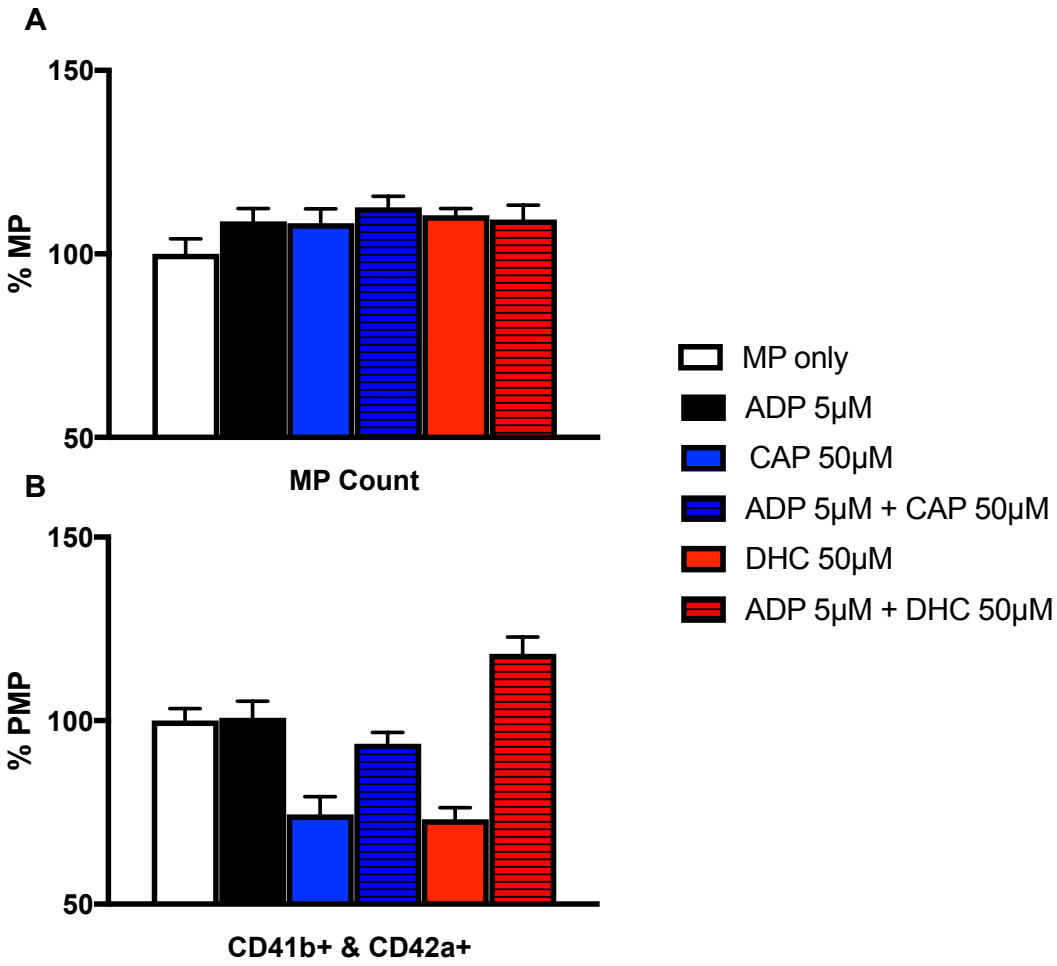

Figure 3-5 (A) The percent of MP in <1 µm gate of MP plasma only, ADP-activated platelets, capsaicin (CAP), dihydrocapsaicin (DHC), ADP+CAP and ADP+DHC. (B) The percent of CD41b+ and CD42a+ platelet derived microparticles (PMP) of (A) samples.

Data are presented as percent of MP and PMP normalised to MP plasma only (i.e. 0 µM ADP and vanilloids). Results are the mean ± SEM, n=4.

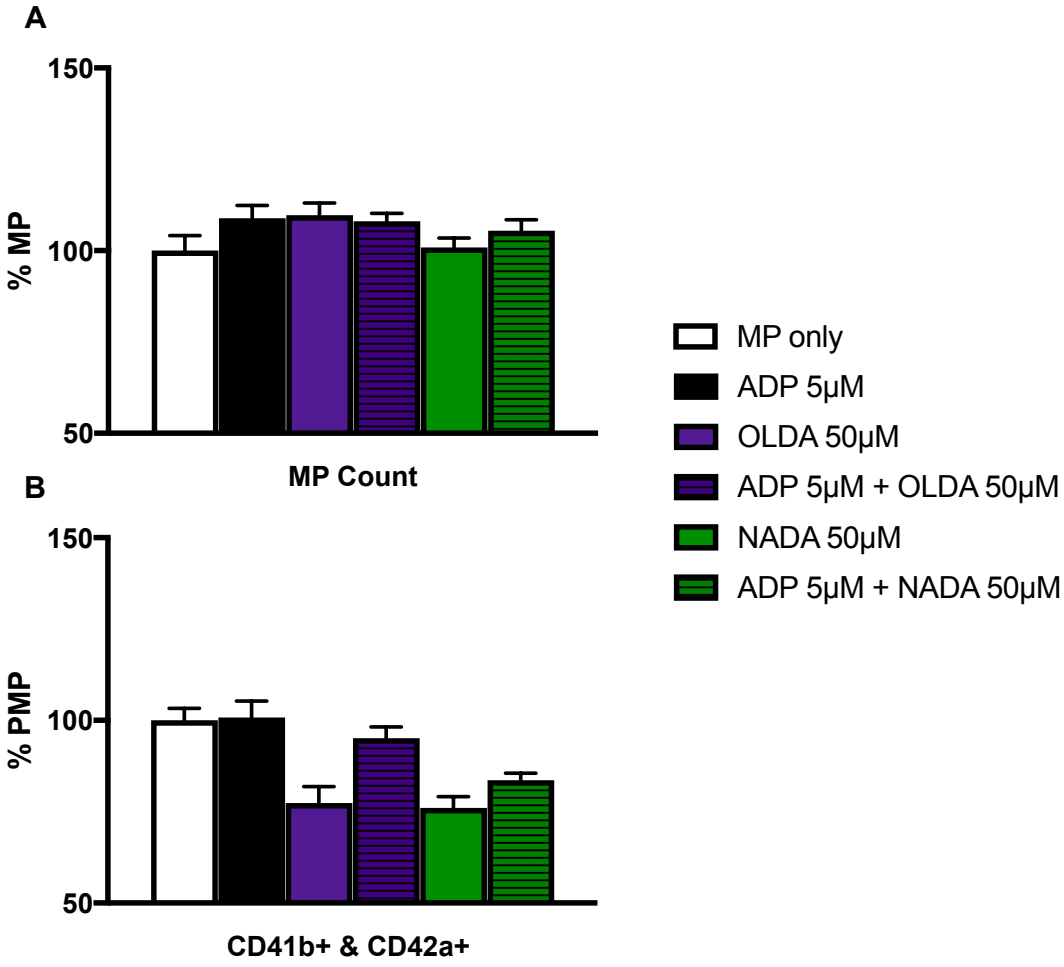

Figure 3-6 (A) The percent of MP in <1 μm gate of MPs plasma only, ADP-activated platelets, N-oleoyldopamine (OLDA) and N-arachidonoyl-dopamine (NADA), ADP+OLDA and ADP+NADA. (B) The percent of CD41b+ and CD42a+ platelet derived microparticles (PMP) of (A) samples.

Data are presented as percent of MP and PMP normalised to MP plasma only (i.e. 0 μM ADP and vanilloids). Results are the mean ± SEM, n=4.

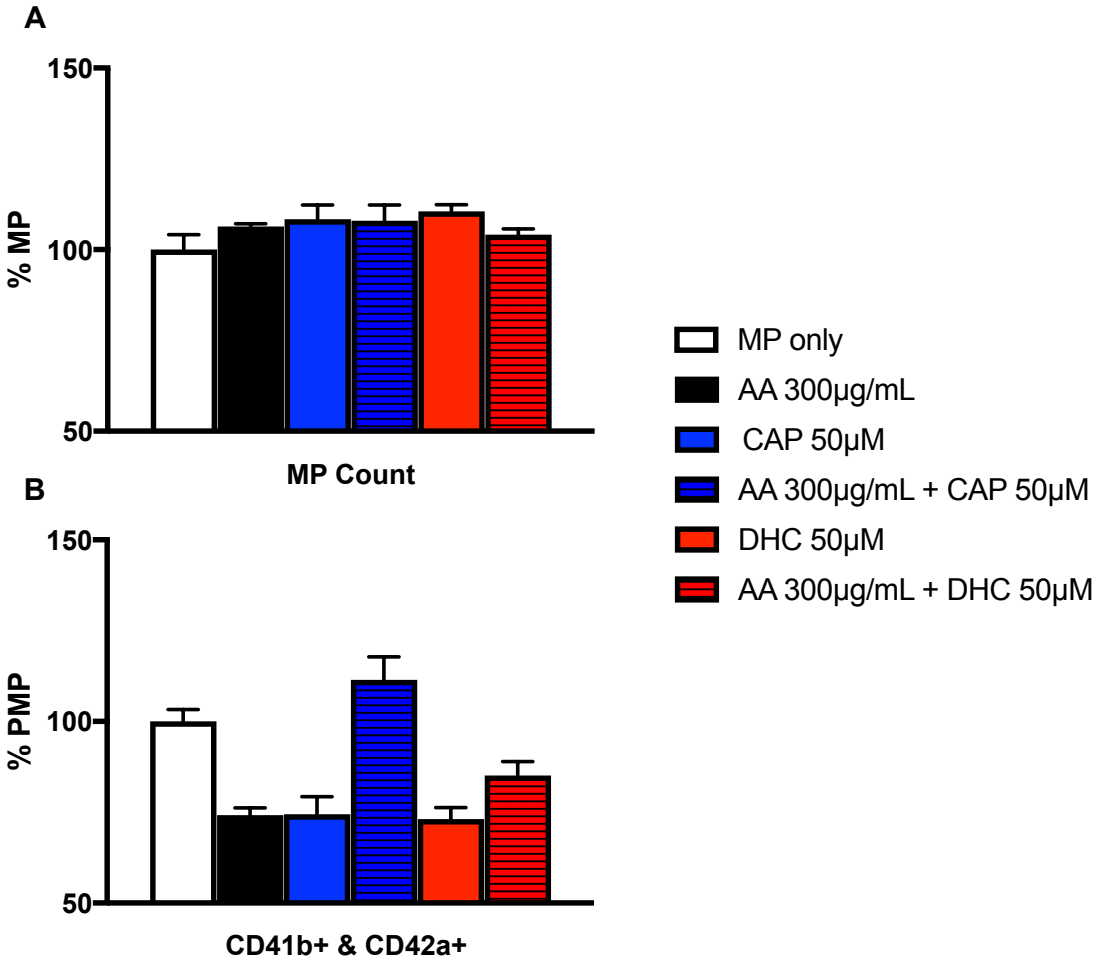

Figure 3-7 (A) The percent of MP in <1 µm gate of MP plasma only, AA-activated platelets, capsaicin (CAP), dihydrocapsaicin (DHC), AA+CAP and AA+DHC. (B) The percent of CD41b+ and CD42a+ platelet derived microparticles (PMP) of (A) samples.

Data are presented as percent of MP and PMP normalised to MP plasma only (i.e. 0 µM AA and vanilloids). Results are the mean ± SEM, n=4.

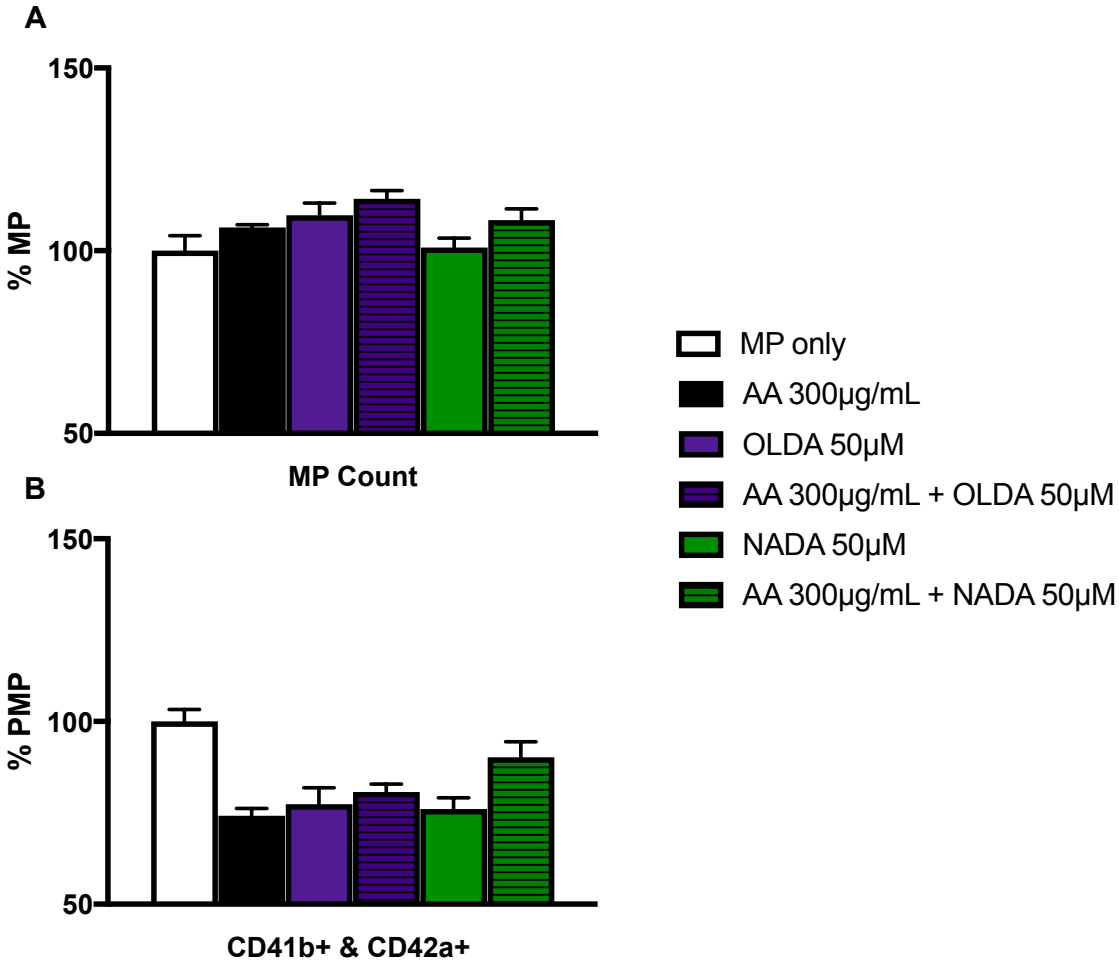

Figure 3-8 (A) The percent of MP in <1 µm gate of MP plasma only, AA-activated platelets, N-oleoyldopamine (OLDA) and N-arachidonoyl-dopamine (NADA), AA+OLDA and AA+NADA. (B) The percent of CD41b+ and CD42a+ platelet derived microparticles (PMP) of (A) samples.

Data are presented as percent of MP and PMP normalised to MP plasma only (i.e. 0 µM AA and vanilloids). Results are the mean ± SEM, n=4.

Table 3-1 Summary of ADP- and AA-generated microparticles (MP) in the presence and absence of vanilloid-like agents, labeled with antibodies to GPIIb (CD41b) and GPIX (CD42a)

	Total % of MP < 1 μm	$CD41b^+/CD42a^-$	$CD41b^-/CD42a^+$	$CD41b^+/CD42a^+$	$CD41b^-/CD42a^-$
MP only	40.2±4.1	27.7±3.2	14.8±2.7	30.2±3.3	27.2±2
ADP	43.7±3.5	19.3±4.5	23.1±3.4	30.4±4.5	27.1±3.8
AA	42.7±0.8	20.3±1.9	22.1±1.7	22.4±2	35.3±0.9
CAP	43.5±3.9	20.3±3.2	25.3±4.8	22.4±4.9	31.8±4
ADP+CAP	45.3±3	17.2±1.9	23.2±2.1	28.3±3.1	31.3±2.5
AA+CAP	43.4±4.4	17.9±4.3	23.8±6.7	33.7±6.4	24.6±4.9
DHC	44.4±1.8	20.8±3.7	30.9±2.8	22.1±3.2	26.2±3.9
ADP+DHC	43.9±3.9	18.8±3.4	18.4±3.4	35.7±4.6	27.1±5.5
AA+DHC	41.8±1.6	20.9±1.7	25.8±3.3	25.7±3.8	27.6±3.5
OLDA	44.1±3.4	24.4±2.2	24.9±1.8	23.4±4.5	27.3±4.3
ADP+OLDA	43.4±2.2	20.2±3	21.6±3.5	28.7±3.1	29.5±5
AA+OLDA	45.9±2.2	21.3±2.9	30.2±0.7	24.4±2.2	24.2±4.1
NADA	40.5±2.6	19.8±3.2	26.7±4	22.9±3	30.5±5.2
ADP+NADA	42.4±3	15.5±2.4	27.2±0.7	25.3±1.9	32.1±2.9
AA+NADA	43.5±3	25.5±2.8	17.7±3.7	27.3±4.2	29.6±3.5

Data are expressed as the percentage of MP <1 μm and fluorescence-positive MP (mean ± SEM; n=4). Variable expression of glycoprotein GPIIb and Integrin alpha IIb, and GPIX were detected in ADP- and AA-derived MP in the presence and absence of vanilloid-like agents, as assessed by CD41b and CD42a, respectively.

3.6. Discussion

The present data suggest that the inhibitory effect of endovanilloids and plant-derived vanilloids on *in vitro* platelet aggregation is caused at least in part through blocking the P_2Y_{12} receptor and inhibiting the AA metabolic pathway, respectively. Plant-derived vanilloids, CAP and DHC, and endogenous vanilloids, NADA and OLDA, did not appear to mediate their inhibitory effects on platelet aggregation through blocking the P_2Y_1 ADP receptor, as dense- and α-granules release were not affected. Finally, the inhibitory effect of vanilloids appears to be independent of changes in PMP formation/release.

The precise mechanism(s) of action of endogenous and plant-derived vanilloids on platelet aggregation remain to be clarified, although numerous hypotheses have been proposed, particularly for CAP. A previous study showed that CAP inhibits cyclooxygenase-1 (COX-1), which may result in inhibition of platelet aggregation (Raghavendra and Naidu, 2009). Furthermore, it has been suggested that CAP inhibits aggregation by inserting into platelet plasma membranes, changing its permeability and fluidity (Hogaboam and Wallace, 1991).

I have previously shown that plant-derived vanilloids and endovanilloids inhibit ADP-induced platelet aggregation, with both vanilloid classes (apart from OLDA) producing a similar effect on AA-induced aggregation (Almaghrabi et al., 2014). These inhibitory effects were not mediated through TRPV1 channels, nor CB1 or CB2 receptors (Chapter 2). Based on these findings, I attempted to determine the mechanism(s) that might explain inhibition of platelet aggregation by vanilloid-like agents. The collagen pathway was not investigated as I have previously shown that

only the endovanilloids, NADA and OLDA, have an inhibitory effect on aggregation induced by a low concentration of collagen (4 μg/mL), which was not observed at the higher concentration (8 μg/mL) (Almaghrabi et al., 2014).

Firstly, VASP phosphorylation status and the measurement of platelet granule release were used to determine whether vanilloids act through blocking the ADP receptors, P_2Y_{12}, and/or P_2Y_1, respectively. It was demonstrated that NADA stimulated phosphorylation of the targeted protein, VASP, by interfering with/blocking the P_2Y_{12} receptor, which leads to increased cAMP formation. To a lesser extent, OLDA showed a non-significant stimulatory effect on VASP phosphorylation. Elevated levels of cAMP interfere with platelet activity by inhibiting fibrinogen receptor activation, cytoskeleton rearrangements, degranulation and expression of pro-inflammatory signaling molecules (Schwarz et al., 2001). In addition, increased cAMP levels are associated with reduced binding ability of thrombin to its receptor (Lerea and Glomset, 1987, Lerea et al., 1987). In my previous study, the effect of CAP was investigated using 5 and 10 μM ADP to stimulate platelet aggregation (Almaghrabi et al., 2014). However, the ADP concentration in VASP kit was unavailable as the company refused to disclose this information. Therefore, the ADP concentration provided in the VASP kit may be higher than 10 μM, which may provide an explanation as to why no effect on VASP phosphorylation in the presence of CAP effect was observed. Moreover, DHC showed no effect on the phosphorylation status of VASP, as it had no effect on ADP-induced aggregation (Almaghrabi et al., 2014). Plant-derived vanilloids and endovanilloids do not seem to exert their action through interference to the ADP receptor, P_2Y_1, as they had no effect on dense- (5-hydroxytryptamine (5-HT)), α-

granules (platelet factor 4 (PF4) or β-thromboglobulin (β-TG)) release.

Subsequently, the possibility that inhibition of platelet aggregation was due to the inhibition of TXA2 production in the AA pathway was investigated. Thromboxane A2 is an important mediator of platelet aggregation and AA markedly increases TXB2, the stable metabolite of TXA2 (Catella et al., 1986). CAP significantly reduced TXB2 formation, in agreement with the study of Raghavendra and Naidu (2009), which reported a significant reduction in TXB2 from washed platelets in the presence of CAP. Moreover, DHC showed non-significant inhibition of TXB2 formation. These results are in agreement with my earlier work as CAP and DHC showed a strong inhibitory effect on AA-induced aggregation (Almaghrabi et al., 2014). OLDA had no effect on TXB2 formation, which was expected since it did not inhibit AA-induced aggregation (Almaghrabi et al., 2014). Whereas NADA showed an inhibitory effect with AA-induced aggregation (Almaghrabi et al., 2014) but not on TXB2 formation. In platelets, AA converts to TXA2 and the latter is a potent inducer of aggregation causing granule release of its contents such as ADP to amplify aggregation. Therefore, NADA may block ADP receptors, P_2Y_{12}, and inhibit platelet aggregation.

Finally, PMP generation increases upon platelet activation, which is associated with increased risk of CVD (Abrams et al., 1990, Morel et al., 2011a, Keuren et al., 2006, Azevedo et al., 2007, Hartopo et al., 2016). Currently used antiplatelet medications, abciximab and clopidogrel, inhibit PMP formation *in vitro* and *ex vivo*, respectively, whereas epoprostenol prevents PMP generation *in vitro* (Reverter et al., 1996, Tamburrelli et al., 2011, VanWijk et al., 2003, Behan et al., 2005). Hence, the effect

of vanilloids on PMP formation/release was investigated in the present study. Contrary to expectations, the presence of vanilloids had an effect, but not statistically significant, on the levels of CD41b/CD42a-positive PMP derived from ADP- and AA-stimulated platelets. Although the results were not significant NADA and OLDA seem to dampen PMP formation in ADP-stimulated platelets. This is noteworthy because NADA is able to inhibit platelet aggregation induced by different agonists (Almaghrabi et al., 2014) and inhibit PMP formation. However, NADA slightly increased PMP formation in AA-stimulated platelets while OLDA showed no effect. Interestingly, the effects of CAP and DHC on PMP formation were non-significantly exacerbated in the presence of the agonists, ADP and AA, which conflicts with their previously demonstrated antiplatelet effects (Adams et al., 2009, Almaghrabi et al., 2014). Increased PMP levels are associated with platelet activation (Abrams et al., 1990, Morel et al., 2011a, Keuren et al., 2006) and involved in the pathogenesis of atherosclerosis and thrombus formation (Tan and Lip, 2005, Lee et al., 1993, Mallat et al., 2000, Lacroix et al., 2013). However, the reason for this effect is unclear and it raises a question about whether or not CAP, DHC and possibly NADA would be safe to use as antiplatelet agents, especially in patients with hyperactive platelets.

Although this study has achieved most of its initial aims, the main limitation was the relatively small sample size. However, the data from this chapter has generated new experimental data on some of the potential mechanisms by which vanilloids inhibit platelet aggregation. In addition, higher concentrations of the platelet agonists, ADP and AA, could have been used in PMP experiments. Concentrations of all agonists and vanilloids were kept consistent across all experiments to allow comparison of the effect of vanilloids on platelets across different studies. Further studies could

include: investigate whether endovanilloids interfere with collagen receptor(s) as they also inhibited collagen-induced platelet aggregation.

In conclusion, plant-derived vanilloids, CAP and DHC, and endogenous vanilloids, OLDA and NADA, inhibited *in vitro* platelet aggregation induced by AA and ADP. NADA and possibly OLDA may exert their inhibitory action through blocking the ADP receptor, P_2Y_{12}, whereas, CAP and possibly DHC via inhibiting AA metabolic pathway. None of the vanilloids inhibited aggregation through the P_2Y_1 ADP receptor, as the release reaction from dense- and α-granules was not affected. Moreover, no changes in the quantity of PMP released in the presence of vanilloids were observed. The mechanism of action of vanilloids should be further investigated as they have a potent antiplatelet effect and appear to inhibit platelet aggregation through different pathways.

4. Chapter 4: Synergistic Inhibitory Effect of Capsaicin and Dihydrocapsaicin on In Vitro Platelet Aggregation and Thromboxane Formation

4.1. Abstract

Capsaicinoids, including capsaicin (CAP) and dihydrocapsaicin (DHC), the pungent principles of pepper fruits, individually inhibit *in vitro* platelet aggregation. However, their effects when present in the relative proportions that they are found naturally, i.e., ~60% CAP and ~40% DHC, are not known. The aims of this study were to compare the effects of CAP and DHC alone, and in combination, on *in vitro* platelet aggregation, platelet count and thromboxane B2 formation (TXB2).

Venous blood was collected from healthy participants in citrate tubes to obtain platelet-rich and platelet–poor plasma. The effects of 12.5 and 6.25 μM CAP and DHC individually, and in combination (CAP:DHC, 60:40) on arachidonic acid (AA) (300 μg/mL)-, ADP (5 μM)-, and collagen (4 μg/mL)-induced *in vitro* platelet aggregation, were investigated. Platelet count was determined pre- and post-incubation with 12.5 μM CAP and DHC, and in combination (60:40 ratio). TXB2 formation from platelets treated with AA (300 μg/mL) in the absence and presence of 12.5 μM and 6.25 μM CAP and DHC individually, and in a 60:40 combination, was measured by ELISA.

Compared to control, CAP and DHC (12.5 μM) inhibited AA-induced aggregation by 23.2% and 25.3%, respectively (both $p<0.01$). In combination, CAP and DHC exhibited further inhibition in AA-induced aggregation (CAP:DHC, 3.75:2.5 μM,

36.5%, p=0.01; CAP:DHC, 7.5:5 μM, 57.5%, p<0.001), compared to control. In contrast to AA-induced aggregation, neither CAP nor DHC individually, or in combination, significantly inhibited ADP- or collagen-induced aggregation. Incubation of platelets with CAP and DHC (12.5 μM), and in combination for up to 2 hours did not significantly affect the platelet count. In addition, the 60:40 CAP:DHC (7.5:5 uM) combination significantly inhibited (p<0.001) TXB2 formation, compared to the individual capsaicinoids.

The present study shows that the combination of CAP and DHC in the proportions they are usually present in pepper fruits, produces a significantly greater inhibitory effect on AA-induced platelet aggregation and subsequent TXB2 formation, compared to the individual capsaicinoids. These results warrant further investigation to determine whether they may be exploited for therapeutic benefit.

4.2. Introduction

Pepper fruits (Capsicum) are one of the most consumed foods worldwide. These fruits contain capsaicinoids, which give them a characteristic pungent taste. Capsaicin (CAP) and dihydrocapsaicin (DHC) are the major capsaicinoids (Bennett and Kirby, 1968, Kosuge and Furuta, 1970) and are usually present in a 60:40 ratio (Garces-Claver et al., 2006) (Table 4-1).

Individually, CAP and DHC have strong inhibitory effects on arachidonic acid (AA)-induced *in vitro* platelet aggregation, and to a lesser extent on ADP-induced platelet aggregation (high concentrations 100-25 μM of CAP) (Almaghrabi et al., 2014). In contrast, neither CAP nor DHC appear to have an inhibitory effect on collagen-induced aggregation (Almaghrabi et al., 2014). The inhibitory action of capsaicinoids

on platelet aggregation could potentially be applied to the prevention and/or treatment of thrombosis and atherosclerosis.

Previous studies investigated the effects of CAP and DHC individually on platelet aggregation (Almaghrabi et al., 2014, Adams et al., 2009, Raghavendra and Naidu, 2009). The aim of this study was to determine the effect of CAP and DHC individually, and in combination (in the relative proportions found in pepper fruits), on *in vitro* platelet aggregation, platelet count and TXB2 formation.

Table 4-1 Concentrations of Capsaicin and Dihydrocapsaicin in Different *Capsicum* Genotypes.

Determined by the HPLC-ESI/MS(TOF) Method Developed [Values Are Means ± SE (n) 3)] (Garces-Claver et al., 2006).

Genotype	Capsaicin (mg kg^{-1} of dry weight)	Dihydrocapsaicin (mg kg^{-1} of dry weight)
Yolo Wonder	Not detected	Not detected
Agridulce	8.7 ± 0.1	8.3 ± 0.8
Sweet Chinense	16.5 ± 0.2	12.9 ± 1.8
Mild Habanero	17.7 ± 1.6	13.4 ± 2.4
Perennial	164 ± 7	192 ± 8
Tabasco	3785 ± 235	2461 ± 80
Orange Habanero	6639 ± 348	3727 ± 179

4.3. Materials

ADP, AA and collagen were purchased from Helena Laboratories (Beaumont, Texas, USA). Capsaicin was obtained from Tocris (Bristol, UK). DHC was obtained from Sigma-Aldrich Pty. Ltd (NSW, Australia). The TXB2 ELISA kits were purchased from Abcam (Melbourne, Australia).

4.4. Methods

Ethics

This study was approved by the Tasmanian Health and Medical Human Research Ethics Committee (approval number: H00011414). Informed written consent was obtained from all participants.

Sample collection and processing

Venous blood was collected from four healthy volunteers (aged 18-65 years), who avoided aspirin and antiplatelet medications for at least 10 d, and dietary chilli for at least two days, prior to collection. Samples were centrifuged at 150g for 10 min at room temperature to obtain platelet rich plasma (PRP). The remaining blood was then centrifuged at 2000g for 20 min at room temperature to obtain platelet poor plasma (PPP).

Platelet aggregation

Platelet aggregation was performed as described in Chapter 2 section 2.4. AA (300 μg/mL), ADP (5 μM), and collagen (4 μg/ mL) were added separately to PRP (225 μl) to initiate aggregation in the presence and absence of CAP and DHC. CAP and DHC were investigated individually at 12.5 μM and 6.25 μM (final concentration), and in combination in a ratio of 60:40 (CAP:DHC; 7.5:5 and 3.75:2.5). Aggregation was recorded for 10 min, with aggregometry data generated by the HemoRAM 1.1.0. Software package (Helena Laboratories, Beaumont, Texas, USA). Percentage of area under curve (%AUC) was recorded and normalised to control (i.e. agonist only).

Platelet count

Platelet counts were determined using a Sysmex 1000i analyser (Roche Diagnostics, Sydney, Australia) with PRP adjusted to 250 x 10^9/L using PPP from the same subject. CAP, DHC (both individually tested at 12.5 μM), and a combination of 7.5 μM CAP and 5 μM DHC, were added separately to PRP, and the platelet count measured every 15 min for 2 hours (n = 4 subjects). PRP with buffer was used as a blank. Data are presented as percent of platelet count normalised to platelet count at time zero.

Measurement of thromboxane B2

Thromboxane B2 release from platelets treated with AA (300 μg/mL) in the absence and presence of 12.5 and 6.25 μM of CAP and DHC and a 60:40 combination was measured using a ELISA kit according to manufacturer's instructions (Appendix 7). Platelets were incubated for ten minutes with capsaicinoids. Data are presented as percent of TXB2 concentration normalised to TXB2 concentration of AA only (i.e. 0 μM capsaicinoid).

Statistical analysis

Data were plotted using GraphPad Prism (version 6; San Diego, CA, USA). Data were compared for AUC differences between control, individual capsaicinoids and combination of CAP and DHC using ANOVA/linear regression (Stata version 13, StataCorp, LP, USA). The Holm test was used to adjust p-values for multiple comparisons. P values < 0.05 were considered statistically significant.

4.5. Results

The effect of CAP and DHC on platelet aggregation

Aggregation data (%AUC) was normalised to control, i.e., platelet agonist only. CAP and DHC (12.5 μM) individually inhibited (both $p<0.001$) AA-induced platelet aggregation by 23.2% and 25.3%, respectively (Figure 4-1A). The combination of 7.5 μM CAP and 5 μM DHC produced even greater inhibition (57.5%, $p<0.0001$) compared to the control (Figure 4-1 A). Similarly, the combination of CAP and DHC in 3.75 μM: 2.5 μM, significantly reduced aggregation (36.5%, $p<0.01$) compared to control (Figure 4-1 A). Additionally, the combination of CAP and DHC (7.5:5 μM) inhibited AA-induced aggregation more than CAP or DHC alone, by 34.3% and 32.2% (both $p<0.05$), respectively (Figure 4-1 A).

Although CAP, DHC and the combination of CAP and DHC led to lower ADP-induced platelet aggregation compared to control (Figure 4-1 B), the results were not statistically significant when adjusted for multiple comparisons. Collagen-induced platelet aggregation was not significantly different between control, CAP, DHC, or CAP and DHC in combination (Figure 4-1 C).

The effect of CAP and DHC on platelet count

Incubation of platelets with CAP and DHC (individually at 12.5 μM), and in combination (CAP:DHC, 7.5:5 μM), for 2 h had no statistically significant effect on platelet count relative to buffer control (Figure 4-2).

The effect of CAP and DHC on TXB2 formation

The combination of CAP:DHC (7.5:5 μM) significantly inhibited TXB2 formation by 4.4% ($p<0.001$), compared to the control and individual capsaicinoids (12.5 μM) (Figure 4-3 A). However, 12.5 μM and 6.25 μM of individual CAP and DHC, and the combination (3.75:2.5 μM) had no statistically significant effect on TXB2 formation (Figure 4-3).

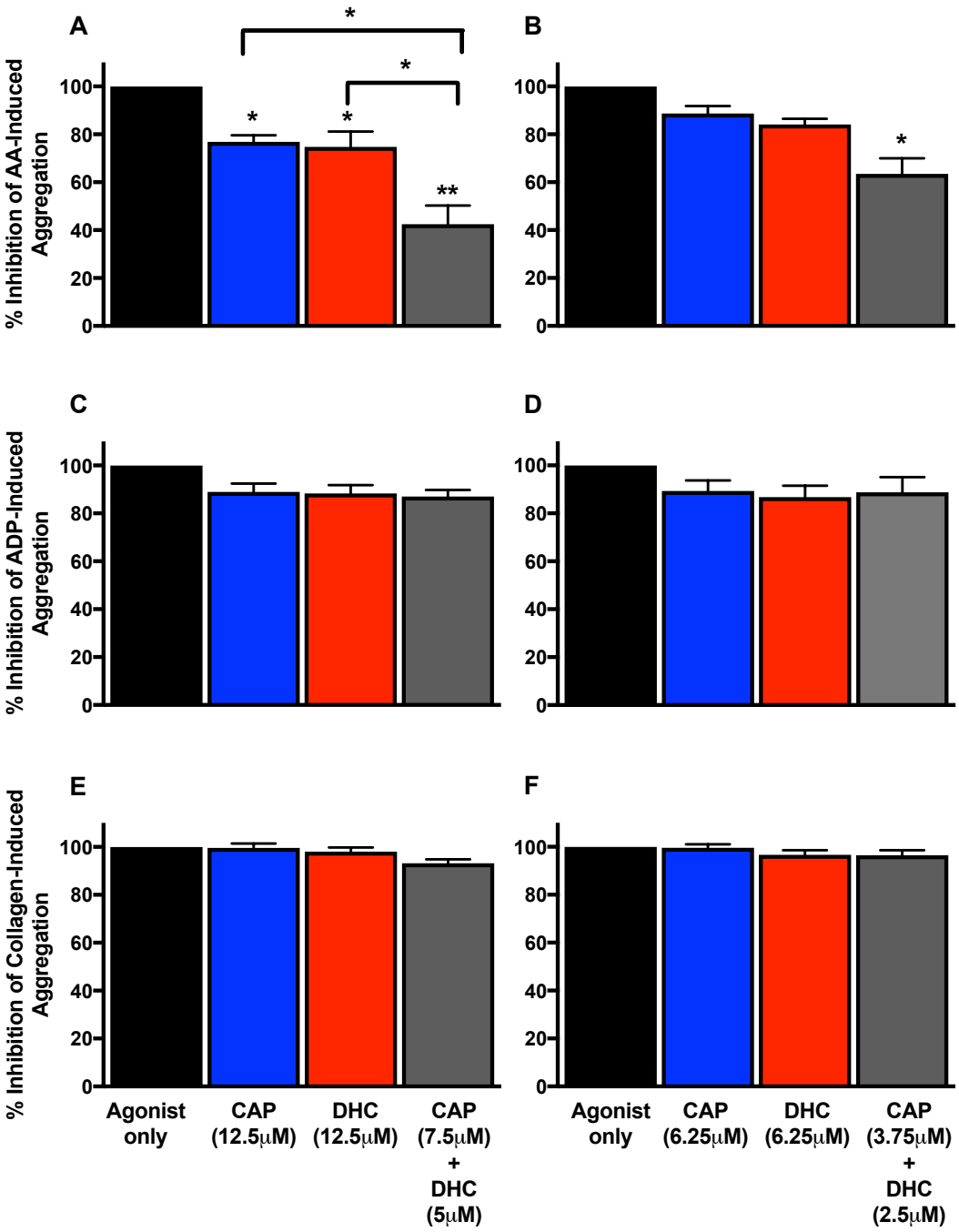

Figure 4-1 The effects of capsaicin (CAP), dihydrocapsaicin (DHC) and their combination on arachidonic acid (AA)- (A and B), ADP- (C and D) and collagen- (E and F) induced platelet aggregation.

Data are presented as percent area under curve normalised to aggregation in the absence of capsaicinoid. Results are the mean ± SEM of 4 experiments. *$p<0.05$, **$p<0.001$ compared to control (i.e. 0 μM of capsaicinoid) and to each column.

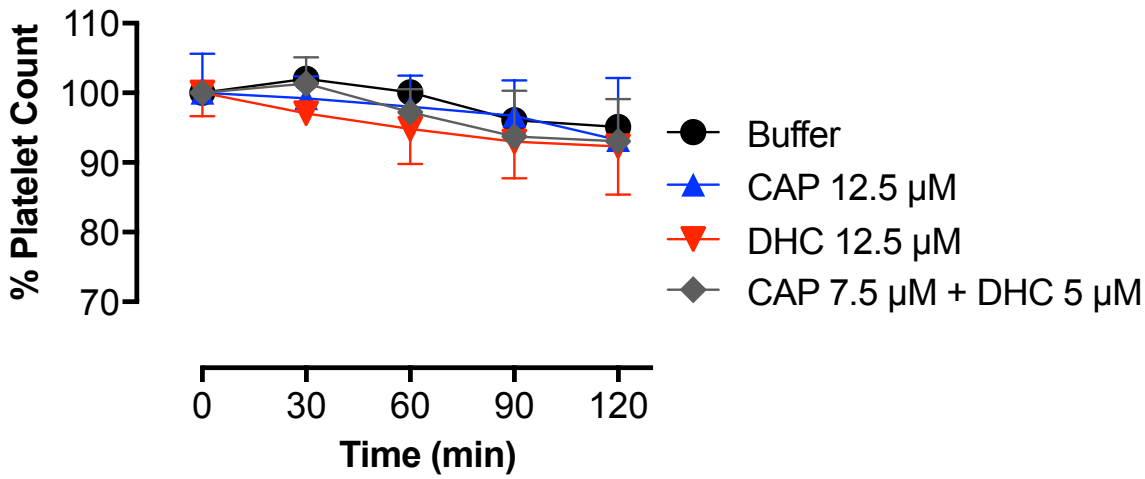

Figure 4-2 12.5 µM of CAP, DHC, and the combination of CAP:DHC (60:40) have no effect on platelet count over 2 hours of incubation.

Data are presented as percent of platelet count (normalised to platelet count at time zero). Mean ± SEM; n = 4.

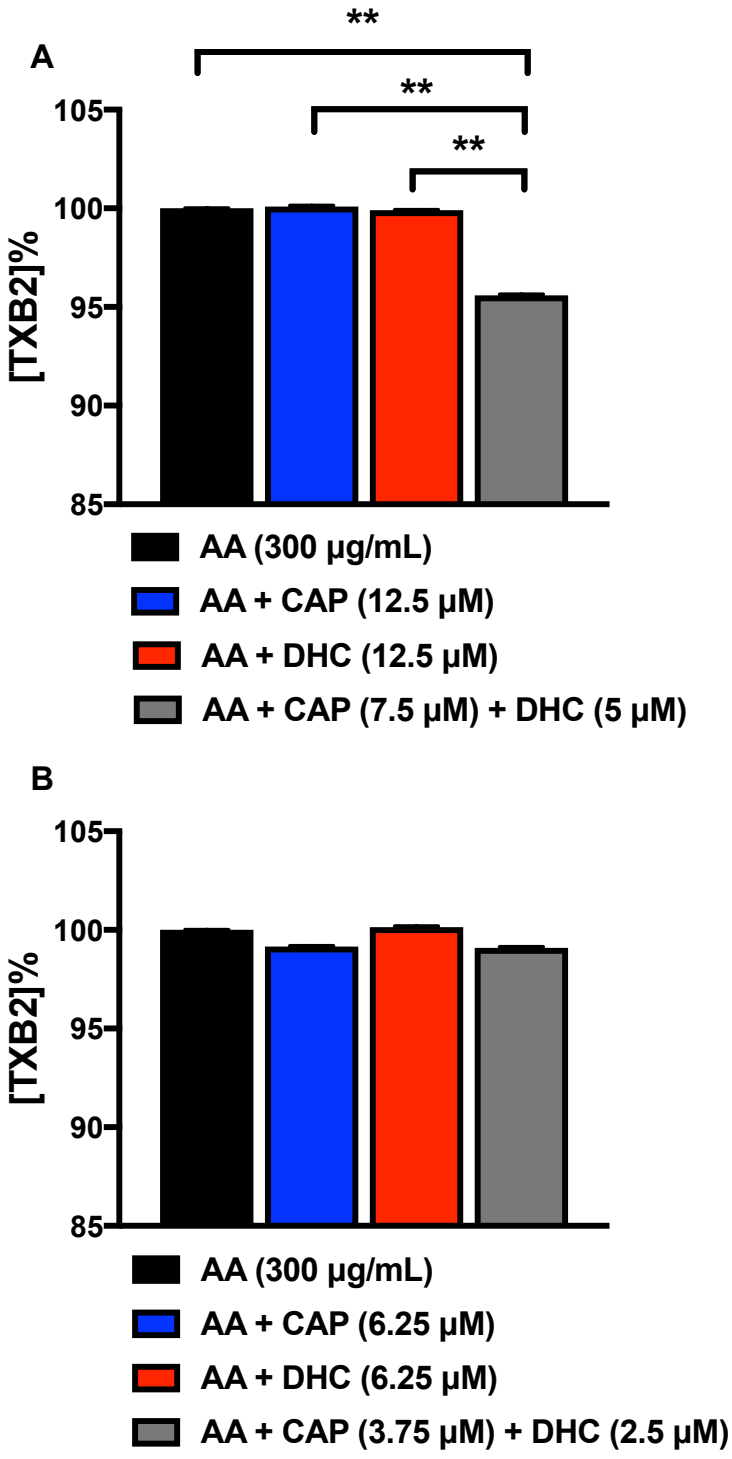

Figure 4-3 The effects of capsaicin (CAP), dihydrocapsaicin (DHC) and their combination on TXB2 formation.

Data are presented as percent of TXB2 concentration normalised to TXB2 concentration with AA only (i.e. 0 µM capsaicinoid). Results are the mean ± SEM, n=4. **p<0.001 compared to control (i.e. 0 µM of capsaicinoid) and to each column. Some error bars are too small to be seen.

4.6. Discussion

This study demonstrated that the combination of CAP and DHC produces greater inhibition of *in vitro* AA-induced aggregation compared to either agent alone, suggesting that these molecules act synergistically. The combined effects of CAP and DHC were greater at lower concentrations, than those used for CAP and DHC individually. In contrast, at the concentrations tested, CAP and DHC individually, and in combination, had no significant effect on ADP- or collagen-induced aggregation. The capsaicinoids and their combination used in this study had no direct effect on platelet count. Furthermore, it was demonstrated that the combination of CAP and DHC act through inhibition of the AA pathway, as TXB2 formation decreased significantly in the presence of both CAP and DHC.

The inhibitory effects of CAP and DHC (individually) on platelet aggregation demonstrated in the current study are similar to what have previously been reported by our laboratory, in that they are concentration dependent (Almaghrabi et al., 2014, Adams et al., 2009) and not due to direct toxicity of CAP and DHC on platelets (Almaghrabi et al., 2014). Although inhibition of *in vitro* platelet aggregation by capsaicinoids has been observed using agonists including AA, ADP and collagen, the degree of inhibition is dependent on the agonist used (Raghavendra and Naidu, 2009, Almaghrabi et al., 2014, Adams et al., 2009). For example, 12.5 μM CAP and DHC individually inhibited AA-induced aggregation (Almaghrabi et al., 2014), whereas ADP-induced aggregation was inhibited at concentrations greater than 25 μM (Almaghrabi et al., 2014, Adams et al., 2009). Similarly, the inhibitory effect of CAP on collagen-induced aggregation varies across studies, which may be due to differences in CAP concentration, the species source of platelets, and/or

methodological differences, e.g., incubation times (Almaghrabi et al., 2014, Raghavendra and Naidu, 2009). Moreover, collagen is a more potent agonist than AA or ADP (Holmsen, 1977) and so may require higher concentrations of CAP or DHC than those used in the current study to produce inhibition of platelet aggregation *in vitro*.

The combination of CAP and DHC had a greater inhibitory effect on AA-induced platelet aggregation and TXB2 formation, compared to CAP and DHC individually. Our data supports the finding that the inhibitory effect of these capsaicinoids may be through suppression of thromboxane A2 synthesis in the AA pathway (Raghavendra and Naidu, 2009). This was reflected by a 70% reduction in thromboxane B2 (the stable metabolite of thromboxane A2) synthesis from washed platelets in the presence of 15 μM CAP (Raghavendra and Naidu, 2009).

There are two possible explanations for the enhanced antiplatelet effect of combined CAP and DHC on the AA pathway. First, each capsaicinoid in the combination may inhibit different enzymes in AA pathway, for example cyclooxyganase-1 and/or thromboxane synthase, which may lead to decreased TXA2 formation and subsequently TXB2. An alternative explanation may be that CAP and DHC act synergistically on one of the enzymes to inhibit TXA2 formation. However, the exact mechanism of the synergistic inhibitory effect of combined CAP and DHC is unknown and warrants further investigation.

The effect of CAP on the gastrointestinal tract has been extensively investigated due to the perception that chilli causes gastric injury and peptic ulcers. However, recent

research challenges this perception as low dose CAP has a gastro-protective effect through increased mucosal secretion, and prevention of gastric mucosal damage caused by indomethacin, ethanol and /or aspirin (Mozsik, 2014, Szabo et al., 2013, Yeoh et al., 1995). Moreover, it has been reported that individuals who regularly consume chilli have a lower incidence of peptic ulcers than those who eat chilli less frequently (Kang et al., 1995).

Aspirin is the most commonly used anti-platelet therapy, but causes side effects such as increased gastric injury and bleeding (Singh and Triadafilopoulos, 1999, Derry and Loke, 2000). However, combined intake of chilli and aspirin was associated with lower gastric injury, compared to aspirin alone, as detected by endoscopic tests performed six hours following their ingestion (Yeoh et al., 1995). Although CAP inhibits *in vitro* platelet aggregation (Adams et al., 2009, Almaghrabi et al., 2014, Raghavendra and Naidu, 2009), a recent study reported no additional inhibition across a 24 hour period in participants who ingested aspirin with CAP, compared to those who were given only aspirin (Sandor et al., 2014). In light of our results in the current study showing enhanced inhibition of platelet aggregation with a combination of CAP and DHC, and studies indicating the beneficial effects of chilli peppers on the gastric mucosa (Yeoh et al., 1995, Kang et al., 1995), it will be prudent to investigate the combined effects of CAP, DHC (or chilli) and aspirin, against aspirin only, on platelet aggregation.

In conclusion, combining CAP and DHC in proportions found in pepper fruits, led to a greater inhibition of AA-induced platelet aggregation and TXB2 formation, compared to individual agents. Moreover, this combination has no effect on platelet

count. To my knowledge, this is the first study to report synergistic effects of a mixture of capsaicinoids on platelet aggregation. Further investigations are warranted to determine the *in vivo/ex vivo* effect of the chilli pepper on platelet aggregation and to assess whether it may be exploited for therapeutic benefit.

5. Chapter 5: The Effects of Vanilloid-like Agents on Platelet Aggregation from Patients with Systemic Lupus Erythematosus: A Pilot Study

5.1. Abstract

Patients with systemic lupus erythematosus (SLE) have a higher risk of thrombotic events and cardiovascular disease (CVD) than the general population and are associated with poorer health outcomes. The risk of thrombosis is decreased with antiplatelet medication. We have found that vanilloid-like agents, including plant-derived vanilloids, capsaicin (CAP) and dihydrocapsaicin (DHC), and endogenous vanilloids, N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA), inhibit *in vitro* platelet aggregation in healthy individuals. Plant-derived vanilloids may decrease the risk of thrombosis and CVD. The aim of this pilot study was to investigate the effects of a range of vanilloid-like agents on *in vitro* platelet aggregation using platelets isolated from patients with SLE.

Venous blood was collected from five female patients previously diagnosed with SLE. ADP (5 μM), collagen (4 μg/mL), and arachidonic acid (AA) (300 μg/mL)-induced platelet aggregation was determined using platelet rich plasma (PRP; $250x10^9$/L) in the absence and presence of the plant-derived vanilloids (CAP and DHC) and the endocannabinoid/endovanilloid agents (OLDA and NADA). Maximum aggregation (%MAX), area under curve (%AUC) and slope of platelet aggregation were determined.

Capsaicin, DHC, OLDA and NADA had no effect on ADP-induced aggregation. Similar results were observed with collagen for CAP, DHC and OLDA. However,

NADA had a significant inhibitory effect on collagen-induced aggregation (0 vs 50 μM; %MAX, 100±7.7% vs 62.2±9.7%, $p<0.001$; %AUC, 100±6.5% vs 77.5±7.8%, $p<0.001$). Arachidonic acid failed to induce aggregation, as all patients were on aspirin and/or other non-steroidal anti-inflammatory drugs (NSAID). The present study using SLE patients' platelets shows that NADA inhibits *in vitro* collagen-induced aggregation, while CAP, DHC and OLDA have no effect on ADP- and collagen-induced aggregation. Determining the effect of NADA on a larger number of SLE patients and further investigation on how to increase its formation/secretion, as an endogenous vanilloid, is warranted.

5.2. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune connective tissue disease, characterised by the production of antibodies to cell nucleus components. Some of these autoantibodies form immune complexes that are responsible for tissue inflammation that directly contribute to the development of different clinical manifestations of the disease (Ruiz-Irastorza et al., 2001). Patients with SLE present with various symptoms, such as arthritis, rash, thrombocytopenia, anemia, nephritis and psychosis. Despite frequently developing thrombocytopenia, SLE patients paradoxically have a higher risk of thrombotic complications, which further increases in the presence of antiphospholipid antibodies (West and Johnson, 1988). Atherosclerosis and CVD are also well-described clinical manifestations (Ward, 1999, Gladman and Urowitz, 1987). Indeed, the incidence of myocardial infarction in SLE is 50-fold higher compared to sex- and age-matched healthy individuals (Manzi et al., 1997).

Platelets have a proinflammatory role in atherosclerosis, and in SLE are abnormally activated and release inflammatory mediators into the local environment, as well as change the adhesive and chemotactic properties of endothelial cells (Gawaz et al., 2005). In addition, systemic inflammation in SLE can modify thrombotic responses by down-regulating anticoagulants, up-regulating procoagulants and inhibiting fibrinolysis, which may individually or collectively increase the risk of thrombus formation (Dahlback, 2012, Esmon and Esmon, 2011, Xu et al., 2010).

Antiplatelet medications have proven effective in the prevention of thrombotic events (Vandvik et al., 2012) with aspirin prescribed to SLE patients for primary prophylaxis (Arnaud et al., 2015, Arnaud et al., 2014). However, aspirin use is associated with increased gastric injury and bleeding (Singh and Triadafilopoulos, 1999, Derry and Loke, 2000, Ivey et al., 1980, Weil et al., 1995). Several studies have shown that the naturally occurring vanilloid in the chilli pepper, capsaicin (CAP), inhibited *in vitro* aggregation of platelets from healthy humans, dogs and rabbits (Raghavendra and Naidu, 2009, Mittelstadt et al., 2012, Almaghrabi et al., 2014, Adams et al., 2009, Hogaboam and Wallace, 1991). In contrast to aspirin, CAP has been shown to have a gastro-protective effect against aspirin-, ethanol-and indomethacin-induced gastric mucosal damage (Mozsik, 2014, Szabo et al., 2013, Yeoh et al., 1995). Furthermore, people who consume chilli on a regular basis have a lower incidence of peptic ulcer (Kang et al., 1995). In addition to CAP, there are other vanilloid-like agents including, the plant-derived vanilloid, dihydrocapsaicin (DHC) an analogue of CAP, and endogenous vanilloids, N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA). Previously, we have found that both plant-derived and endogenous vanilloids inhibit *in vitro* platelet aggregation in healthy individuals (Adams et al., 2009, Almaghrabi et al., 2014). Whereas, to my

knowledge, no studies have been published that have investigated the effect of vanilloid-like agents on platelets from patients with high risk of atherosclerosis and thrombosis. The aim for this study was to therefore investigate the effects of plant-derived and endogenous vanilloids on *in vitro* aggregation of platelets collected from patients with SLE.

5.3. Materials

Adenosine diphosphate (ADP) and collagen were obtained from Helena Laboratories (Beaumont, TX, USA). CAP, NADA and OLDA were obtained from Tocris (Bristol, UK). DHC was obtained from Sigma-Aldrich (St Louis, USA).

5.4. Methods

Ethics

This study was approved by the Tasmanian Health and Medical Human Research Ethics Committee (approval number: H00011414). Informed written consent was obtained from all participants.

Sample collection and processing

The volunteers were five females (aged 48–75 years) diagnosed with SLE, who avoided dietary chilli for at least 2 days before providing blood samples. All subjects were taking different combinations of medications such as non-steroidal anti-inflammatory drugs (NSAID), anti-malarial, chemotherapeutics and immune system suppressants. Venous blood was collected by venipuncture using minimal stasis from the patients. Whole blood (20 mL) was collected into 3.2% sodium citrate

anticoagulant tubes (1:9 ratio of anticoagulant to whole blood). Samples were centrifuged at 150g for 10 minutes at room temperature to obtain PRP. The remaining blood was then centrifuged at 2000g for 20 minutes at room temperature to obtain platelet poor plasma (PPP).

Preparation of vanilloid-like agents

CAP, DHC and OLDA were dissolved in 100% ethanol and stored as 0.1M aliquots at -20°C. NADA was stored as 11.37 x 10^3μM aliquot at -20°C. Aliquots of all vanilloids were diluted as required in normal buffered saline (pH 7.1) to provide final incubation concentrations ranging from 3.125-50 μM.

Preparation of platelet aggregation agonists

ADP and AA were dissolved in deionised water and stored in 1M aliquots at -20°C until required. Collagen was provided as ready to use and stored at 4°C. Aliquots of agonists were thawed and diluted in normal buffered saline (pH 7.1) to produce final concentrations; ADP (5 μM), collagen (4 μg/mL) and AA (300 μg/mL).

Platelet aggregometry

Platelet aggregation was performed as described in Chapter 2 section 2.4. PRP (225 μL) was activated with 25 μL of ADP (5 μM) or collagen (4 μg/mL), in the presence and absence of CAP, DHC, NADA or OLDA (final concentrations, 3.125-50 μM). Aggregometry data were generated by the HemoRAM 1.1.0. software package (Helena Laboratories, Beaumont, USA). Parameters included maximum percent of aggregation (%MAX, reflecting the intensity of aggregation responses) percent of

area under the curve (%AUC), slope (rate of aggregation) and lag time per second (reflecting the time from addition of the agonist until the aggregation commenced). These parameters of ADP-, and collagen-induced aggregation were normalised to control (i.e. agonist only). Each data set is presented as the mean of five experiments, using platelets collected from five separate volunteers.

Statistical analysis

Data were plotted using GraphPad Prism (version 7; San Diego, CA, USA). All data were analysed statistically with repeated measures ANOVA using general linear modelling (Stata version 13, StataCorp, LP, USA). Post-estimation Holm test analysis was then used to adjust p values for multiple comparisons. P values < 0.05 were considered significant.

5.5. Results

Summarised data for the effects of plant-derived vanilloids and endovanilloids on SLE patients' platelet aggregation induced by ADP and collagen is presented in Table 5-1. Graphical representation of aggregation data (%MAX, %AUC, slope and lag time) is presented as data normalised to control, i.e., platelet agonist only (Figures 5-1- 5-4).

Effect of vanilloid-like agents on ADP-induced aggregation

Plant-derived vanilloids, CAP and DHC, had no effect on MAX% and %AUC on ADP-induced platelet aggregation in comparison to control (no treatment control) (Figure 5-1). However, the slope of the aggregation curve was enhanced in the presence of CAP (0 vs 50 μM; slope, 100±7.4% vs 122.7±11.9%), but this effect was not statistically significant (Figure 5-1 C,F). Furthermore, the endovanilloids OLDA and NADA had no statistically significant effect on any of the measured parameters of platelet aggregation (Figure 5-2).

Table 5-1 Effect of vanilloids-like agents on ADP-, collagen-induced platelet aggregation from SLE patients.

Agonist					**ADP (5 μM)**			
	CON	**CAP (3.125 μM)**	**CAP (12.5 μM)**	**CAP (50 μM)**	**CON**	**DHC (3.125 μM)**	**DHC (12.5 μM)**	**DHC (50 μM)**
% MAX	61.3±9.2	59.38±8.4	61.12±7.8	59.5±8.1	64.1±7.5	62.4±7.3	63.5±8.1	63.9±6.8
% AUC	48.5±8.3	46.3±8.1	39.1±10.2	46.9±7.5	51.6±6.8	48.3±6.8	50.6±7.5	51.1±6.1
Slope	69.1±7.4	83.1±9.3	81.8±10.5	84.8±11.9	78.5±7.5	86.6±8.4	84.7±8.1	69.1±16.6
Agonist					**ADP (5 μM)**			
	CON	**NADA (3.125 μM)**	**NADA (12.5 μM)**	**NADA (50 μM)**	**CON**	**OLDA (3.125 μM)**	**OLDA (12.5 μM)**	**OLDA (50 μM)**
% MAX	63.9±7.6	61.9±7.6	61.7±8.7	59.4±7.1	61.4±7.5	61.3±7.3	63.3±7.5	63.4±7.1
% AUC	49.8±7.6	48.4±6.8	48.8±7.9	45.9±6.9	48±6.9	48.4±7.2	50.4±7.1	50.2±6.7
Slope	95.6±14.5	100.4±14.9	100.7±18.5	96.5±10.2	95.5±18	86.7±11.7	94.8±13.5	96.6±12.8
Agonist					**Collagen (4 μg/mL)**			
	CON	**CAP (3.125 μM)**	**CAP (12.5 μM)**	**CAP (50 μM)**	**CON**	**DHC (3.125 μM)**	**DHC (12.5 μM)**	**DHC (50 μM)**
% MAX	47.5±10.3	50.5±5.3	51.8±5.7	49.7±5.3	53±7.2	49.5±6.1	51.4±7.3	48.5±6.4
% AUC	42.4±3.3	35.8±4.6	36.9±4.6	36.2±3.9	38.8±6.1	34.7±5.1	36.1±5.7	34.8±5.1
Slope	27±5.4	26.2±4.5	25.1±4.8	24.5±3.5	24.1±5.5	25±4.2	23.9±4.9	20.2±3.6
Lag time/s	6.36±4.8	28.9±10.1	41.9±21	8.6±4.3	36.3±25.4	48.4±24.2	48.3±22.9	29.8±24.7

Agonist	Collagen (4 μg/mL)							
	CON	**NADA (3.125 μM)**	**NADA (12.5 μM)**	**NADA (50 μM)**	**CON**	**OLDA (3.125 μM)**	**OLDA (12.5 μM)**	**OLDA (50 μM)**
% MAX	60.8±7.6	48.5±7.2*	42.8±8.2*	37.8±9.7*	56.1±7.2	53.6±5.1	54.7±5.8	51±6.5
% AUC	44.8±6.5	34.9±5.8*	30.4±6.7*	34.8±7.7*	40.7±5.7	38.5±4.1	39.6±4.9	37.9±5.1
Slope	26.9±5.2	19.8±3.5	19.9±4.7	24.9±6.1	24.8±3.6	25.1±4.6	24.4±7.9	17.3±2.5
Lag time/s	28.9±25.7	29.7±9	50.7±26.6	21.6±11.2	27.3±24.4	25.5±8.7	28.7±9.3	24.7±9.8

Data are presented as percent maximum aggregation (%MAX), percent area under curve (%AUC), slope of the propagation phase of curve (slope) and lag time for collagen-induced aggregation (Lag time/s). Data are the mean ± SEM of 5 experiments for (3.125-50 μM) capsaicin (CAP), dihydrocapsaicin (DHC), N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA). * P <0.001 compared to control (CON) (i.e. 0μM vanilloids).

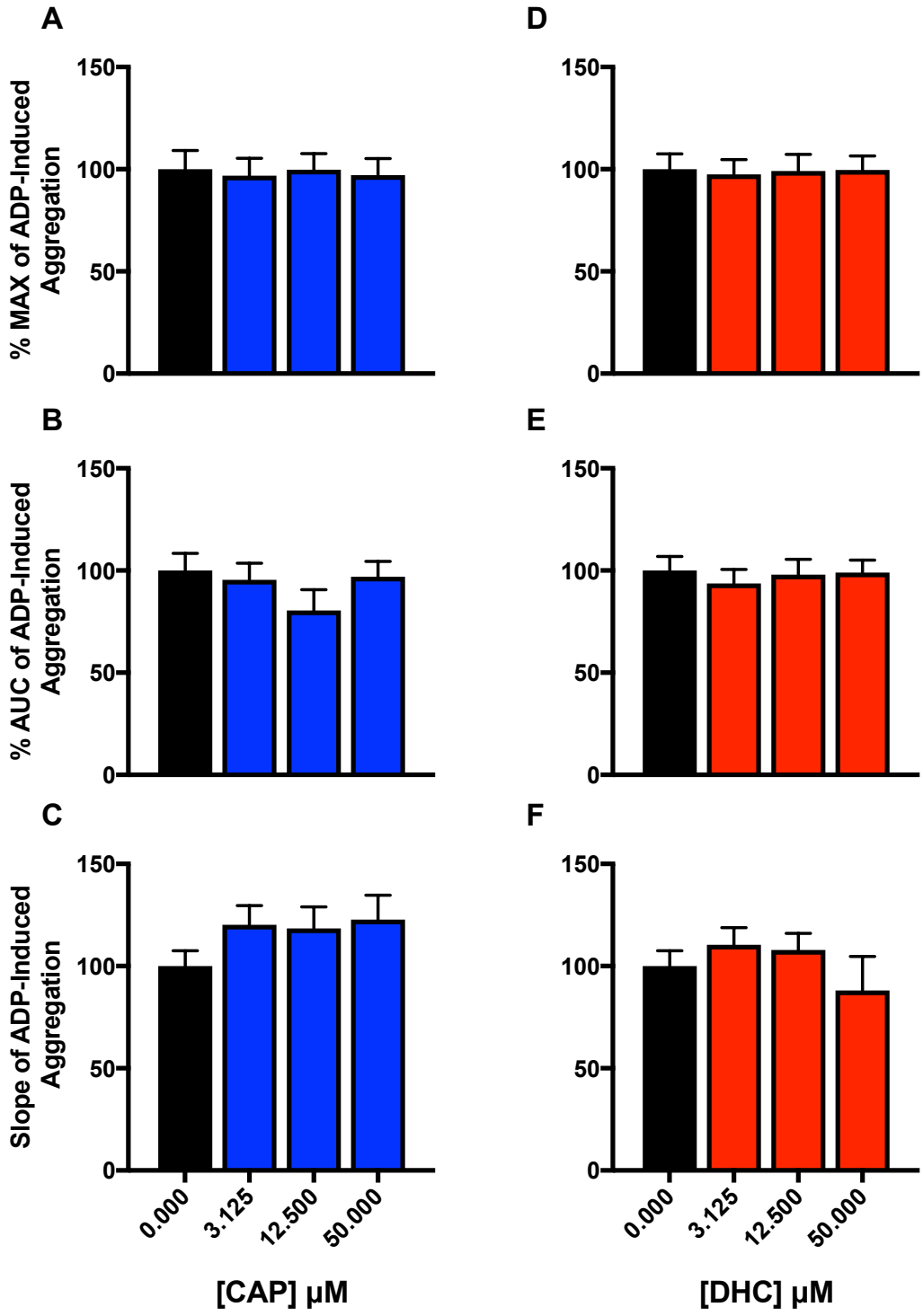

Figure 5-1 Effect of plant-derived vanilloids on 5 µM ADP-induced platelet aggregation in SLE patients.

Data are presented as percent maximum aggregation (%Max, normalised to aggregation in the absence of vanilloid to 100%) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Bars are the mean ± SEM of 5 experiments, for capsaicin (CAP) and dihydrocapsaicin (DHC).

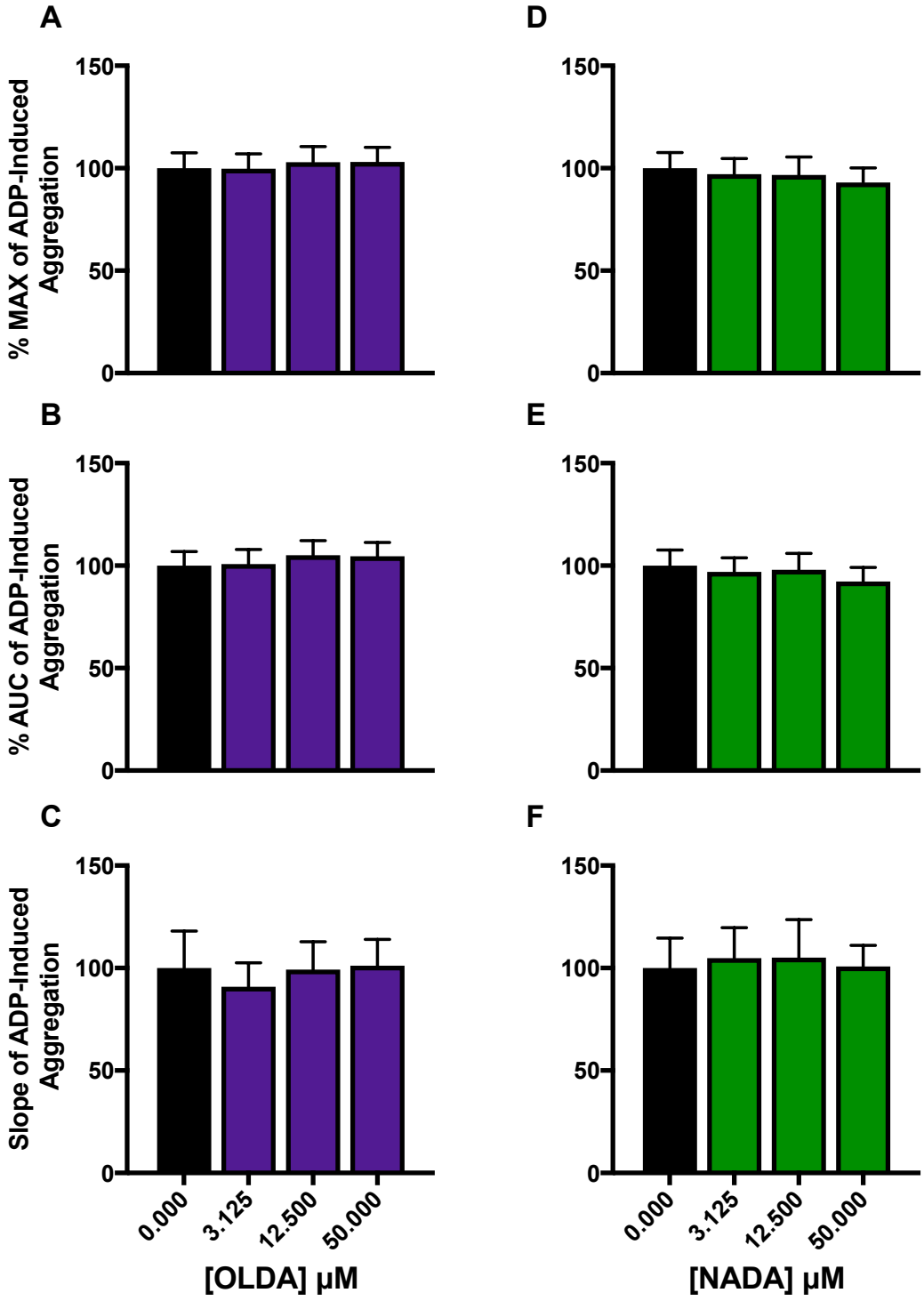

Figure 5-2 Effect of endovanilloids on 5 µM ADP-induced platelet aggregation in SLE patients.

Data are presented as percent maximum aggregation (%Max, normalised to aggregation in the absence of vanilloid to 100%) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Bars are the mean ± SEM of 5 experiments, for N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA).

Effect of vanilloid-like agents on collagen-induced aggregation

CAP and DHC did not affect any of the measured parameters of collagen-induced platelet aggregation (Figure 5-3). However, CAP prolonged the lag time at 3.125 μM and 12.5 μM, of SLE platelets (Figure 5-3 D), although this was not statistically significant.

NADA had a significant inhibitory effect on collagen-induced aggregation (0 vs 50 μM; %MAX, 100±7.7% vs 62.2±9.7%, $p<0.001$); (%AUC, 100±6.5% vs 77.5±7.8%, $p<0.001$) (Figures 5-4 E,F). However, NADA had no significant effect on the slope or lag time of collagen-induced aggregation. Moreover, OLDA did show any statistically significant effect on collagen-induced aggregation measured variables (Figure 5-4).

Effect of vanilloids on platelets from healthy individuals

Contrary to my earlier observations that plant and endogenous vanilloids inhibited agonist induced aggregation of healthy platelets (Almaghrabi et al., 2014), the platelets obtained from SLE patients did not show any change in presence of vanilloids and there were discrepancy in slope and lag time results. To ensure that these results were not due to an experimental error, I repeated the experiment in platelets obtained from four healthy volunteers (three females and one male; aged 32–50 years). Vanilloids had the same inhibitory effect on platelet aggregation (Figure 5-5 and 5-6), as previously reported (Almaghrabi et al., 2014).

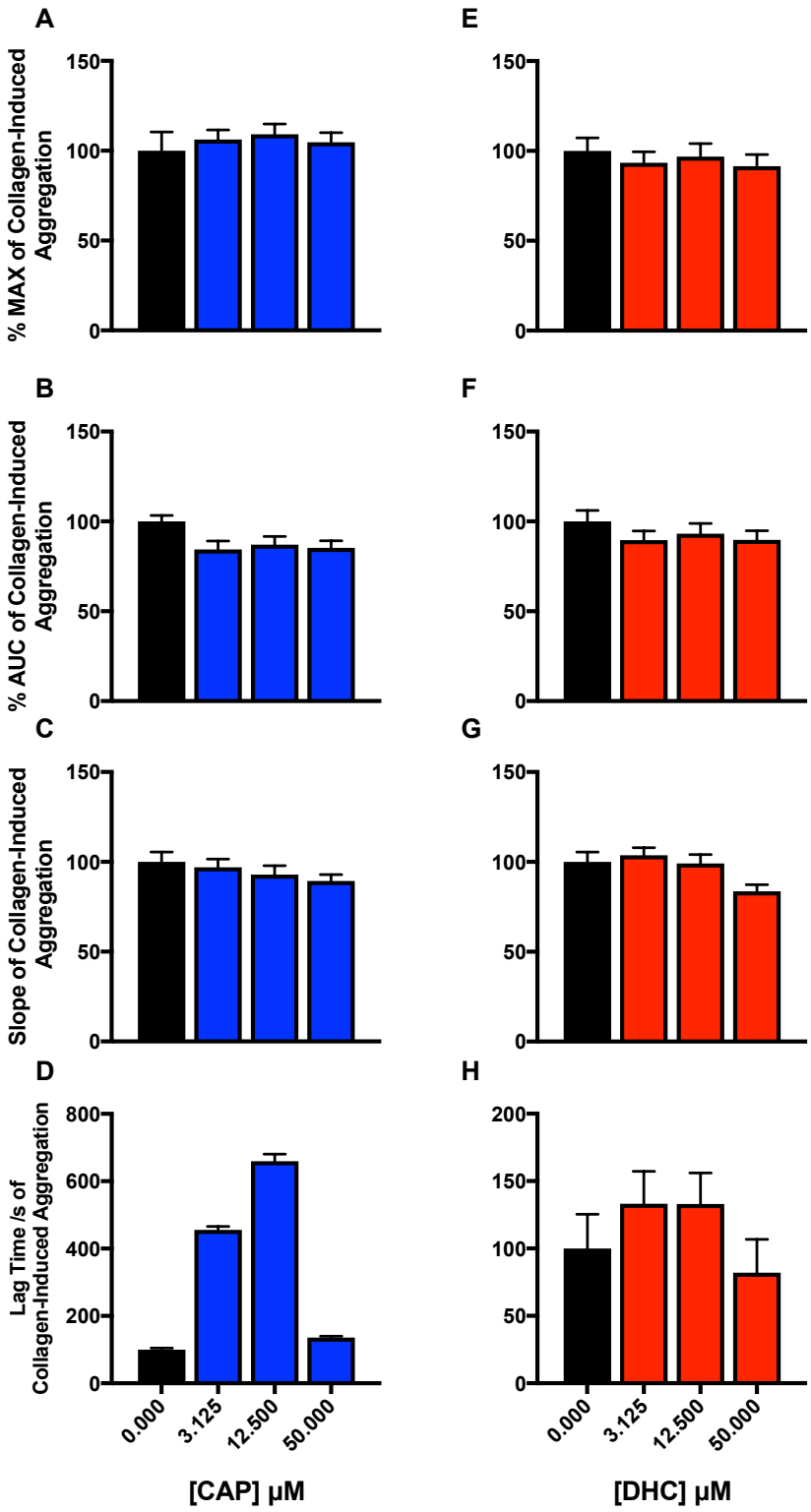

Figure 5-3 Effect of plant-derived vanilloids on 4 µg/mL collagen-induced platelet aggregation in SLE patients.

Data are presented as percent maximum aggregation (%Max, normalised to aggregation in the absence of vanilloid to 100%) (A,E), percent area under curve (%AUC) (B,F), slope of the propagation phase of curve (slope) (C,G) and lag time/s (D,H). Bars are the mean ± SEM of 5 experiments, for capsaicin (CAP) and dihydrocapsaicin (DHC).

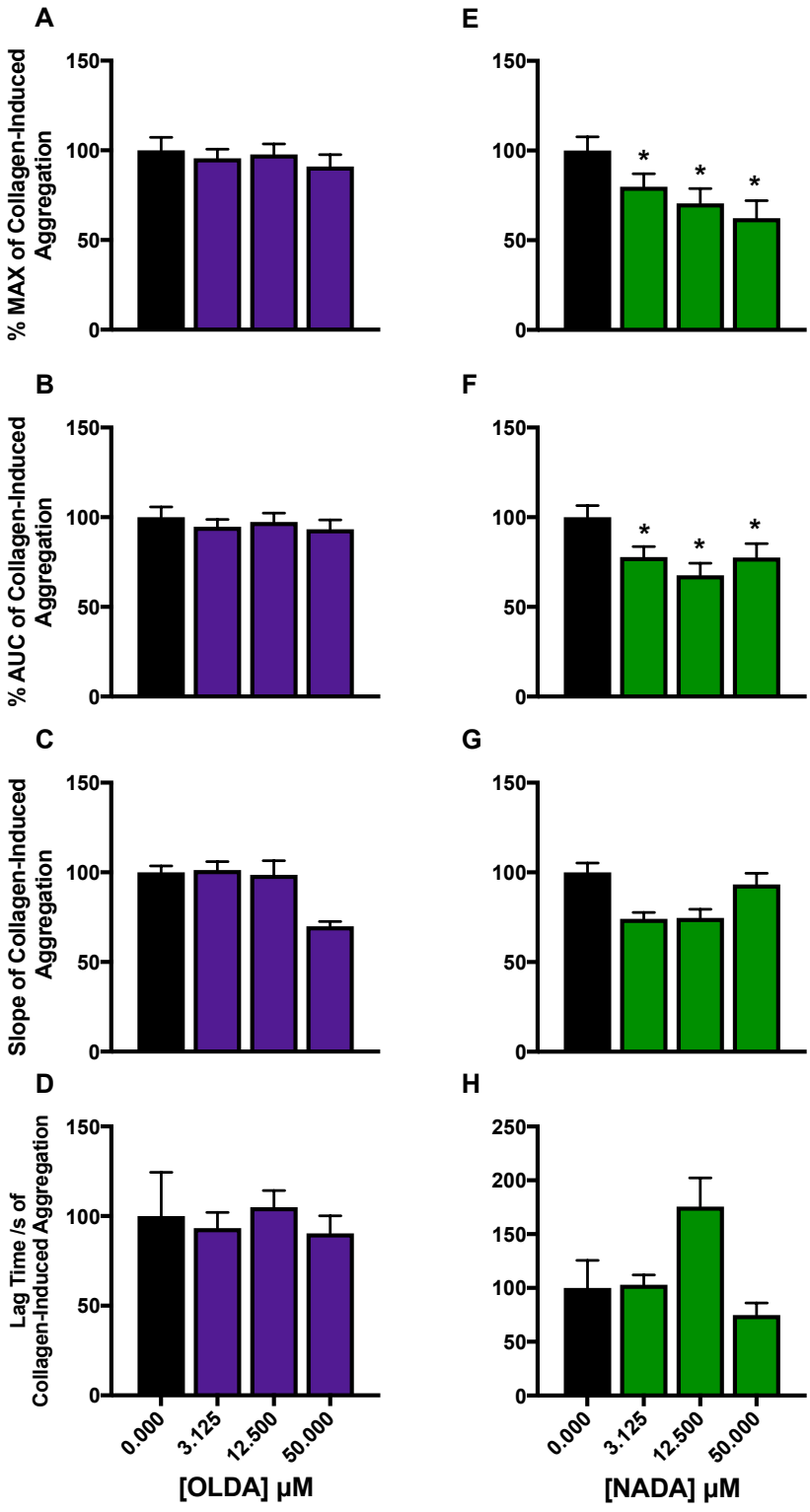

Figure 5-4 Effect of endovanilloids on 4 µg/mL collagen-induced platelet aggregation in SLE patients.

Data are presented as percent maximum aggregation (%Max, normalised to aggregation in the absence of vanilloid to 100%) (A,E), percent area under curve (%AUC) (B,F), slope, of the propagation phase of curve (slope) (C,G) and lag time/s (D,H). Bars are the mean ± SEM of 5 experiments, for N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA). * P <0.05 compared to control (i.e. 0µM vanilloids).

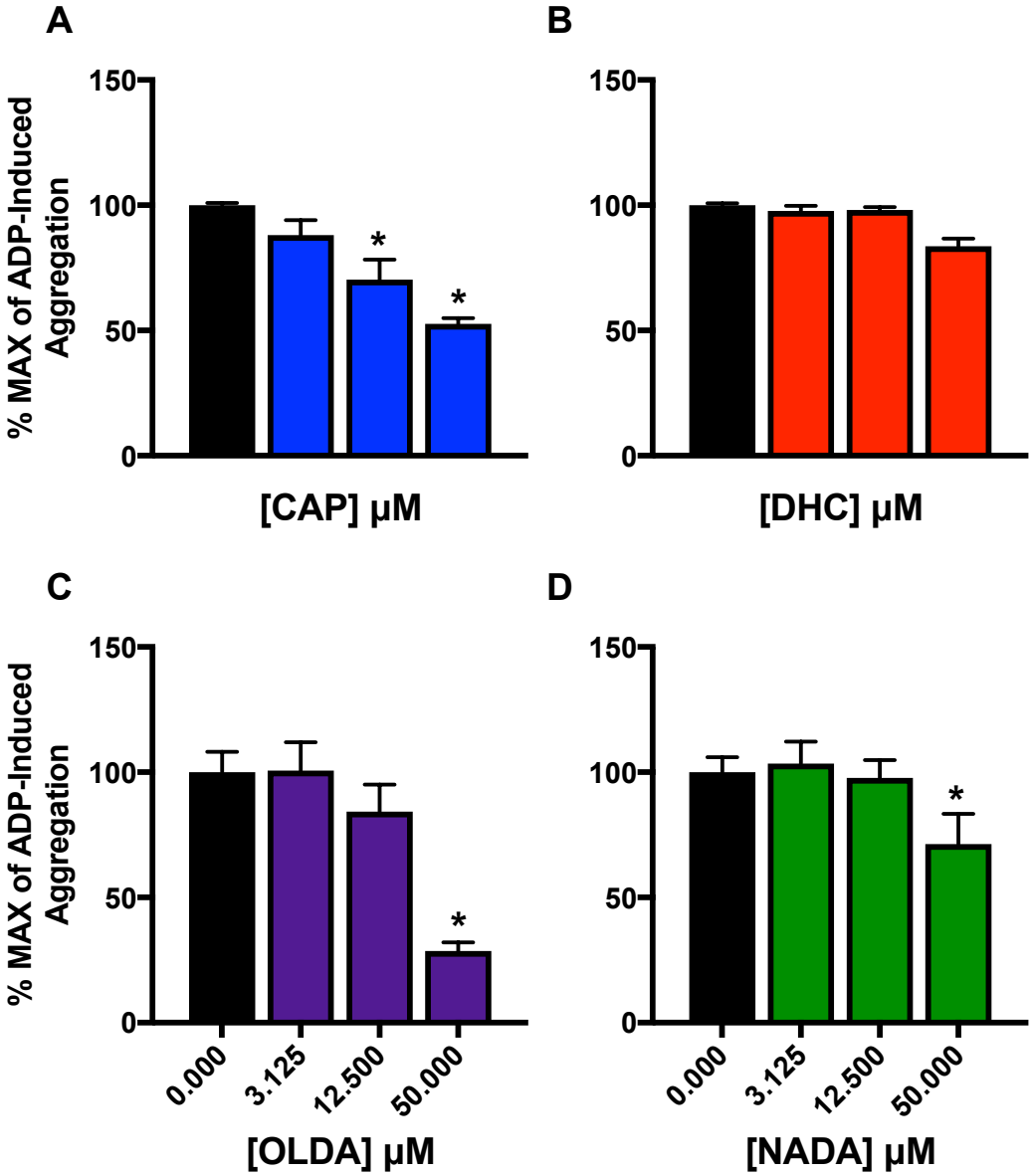

Figure 5-5 Effect of plant-derived vanilloids and endovanilloids on 5 µM ADP-induced platelet aggregation in healthy individuals.

Data are presented as percent maximum aggregation (%Max, normalised to aggregation in the absence of vanilloid to 100%) (A,B,C,D). Bars are the mean ± SEM of 4 experiments, for capsaicin (CAP), dihydrocapsaicin (DHC), N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA). * P <0.05 compared to control (i.e. 0µM vanilloids).

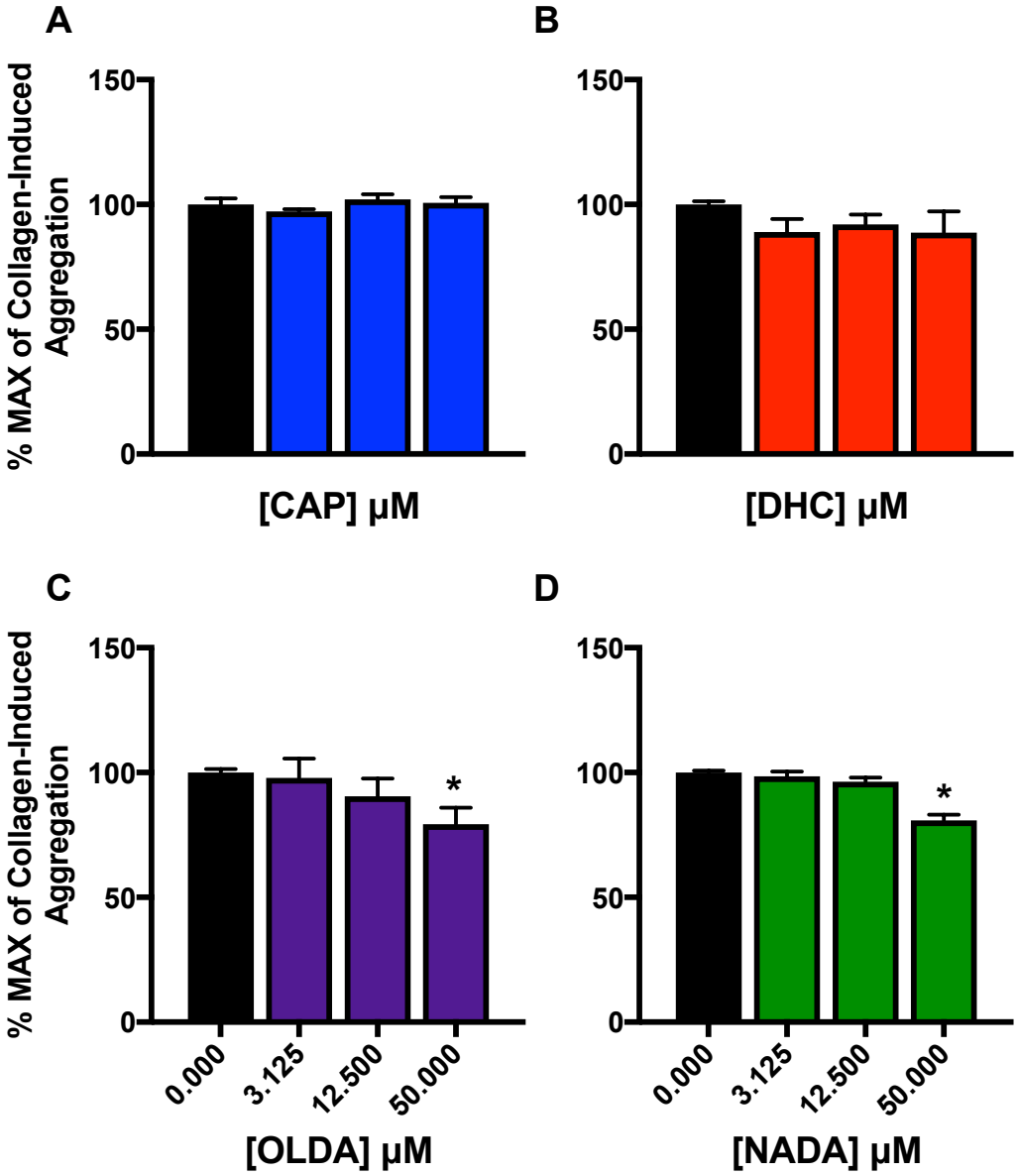

Figure 5-6 Effect of plant-derived vanilloids and endovanilloids on 4 µg/mL collagen-induced platelet aggregation in healthy individuals.

Data are presented as percent maximum aggregation (%Max, normalised to aggregation in the absence of vanilloid to 100%) (A,B,C,D). Bars are the mean ± SEM of 4 experiments, for capsaicin (CAP), dihydrocapsaicin (DHC), N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA). * P <0.05 compared to control compared to control (i.e. 0µM vanilloids).

5.6. Discussion

To my knowledge, this is the first study to investigate the effects of plant-derived vanilloids, CAP and DHC, and endovanilloids, OLDA and NADA, on *in vitro* aggregation of platelets from SLE patients. NADA inhibited collagen-induced aggregation but had no effect on ADP-induced aggregation. Furthermore, CAP, DHC, and OLDA had no effect on *in vitro* ADP- or collagen-induced platelet aggregation. For completion, platelet aggregation was initially investigated using AA as the agonist. However, all SLE patients were taking aspirin and/or other NSAID, and as these medications dampen platelet function through the AA pathway irreversibly inactivating COX-1 enzyme and TXA2 synthesis (Vane, 1971, Atkinson and Collier, 1980, Smith and Willis, 1971), the data for AA-induced *in vitro* aggregation was not included in this chapter.

It has been previously reported that CAP, OLDA and NADA inhibit aggregation induced by ADP using platelets from healthy donors, while only endovanilloids inhibited collagen-induced aggregation (Almaghrabi et al., 2014, Adams et al., 2009). In addition, CAP and DHC, as well as NADA, completely inhibited AA-induced aggregation (Almaghrabi et al., 2014). The anti-aggregating effects of plant-derived vanilloids and endovanilloids were independent of transient receptor potential vanilloid-1 (TRPV1) and cannabinoid (CB1 and CB2) receptors (Chapter 2). Moreover, the effects of both classes of vanilloid-like agents on aggregation were shown not to be due to direct toxicity toward platelets (Almaghrabi et al., 2014). Instead, NADA was found to inhibit platelet aggregation by interfering with the ADP receptor, P_2Y_{12}, as phosphorylation levels of vasodilator-stimulated phosphoprotein (VASP) were significantly increased (Chapter 3).

Determining the effect of vanilloid-like agents on platelets from SLE is complicated as platelets are affected by the disease course itself, and/or medications. Platelets in SLE patients are abnormally activated, as reflected by increased levels of thromboxane B2, surface and soluble P-selectin, circulating platelet-derived microparticles, as well as platelet and/or leukocyte aggregates (Ferro et al., 1999, Nagahama et al., 2001, Tam et al., 2003, Joseph et al., 2001, Sellam et al., 2009). Furthermore, in SLE patients, interferon-α modulates platelet and megakaryocyte activity by up-regulating multiple mRNA and proteins (Lood et al., 2010). Moreover, plasma membranes of platelets isolated from SLE patients have been shown to be 'shaggy and rough', unlike the smooth surface of normal platelets, which is presumably due to immune complex deposition on the surface (Andrianova et al., 2016). Therefore, the pathway(s) through which vanilloid-like agents inhibit platelet aggregation in healthy platelets might be impaired, blocked or altered by SLE itself, or due to medications. In the present study CAP, DHC nor OLDA demonstrated an inhibitory effect toward platelets from SLE patients.

Interestingly, NADA, that appears to act through blocking the P_2Y_{12} receptor in healthy platelets (Chapter 3), inhibited aggregation of platelets from SLE patients in a concentration-dependent manner. Many studies have reported the potential contribution of the P_2Y_{12} receptor in inflammatory diseases (Cattaneo, 2015, Gachet, 2012, Steinhubl et al., 2007, Liverani et al., 2016). Furthermore, blocking this receptor with clopidogrel results in reduced the exposure of P-selectin and CD40 ligand, decreased circulating inflammatory mediators (tumor necrotic factor-α (TNF-α) and C-reactive protein), and inhibits platelet-leukocyte aggregate formation (Cattaneo, 2015, Gachet, 2012, Steinhubl et al., 2007). In addition to the inhibitory

effect on platelet aggregation, NADA has also been shown to reduce the inflammatory response of human endothelial cells induced by exogenous and endogenous inflammatory agonists (Wilhelmsen et al., 2014). Furthermore, NADA inhibited TNF-α and interleukin-2 gene transcription and suppressed the associated signaling pathways, which mediate the activation of some transcriptions factors known to have an important role in the immune system (Sancho et al., 2004). Therefore, NADA may have important anti-inflammatory and anti-platelet properties that could have important implications for helping treat the symptoms of SLE.

Activation of platelets by collagen is a complex process and involves several receptors, including GPIV, integrin K2L1, GPVI, p65 and a TIIICBP type III collagen receptor (Clemetson and Clemetson, 2001, Monnet and Fauvel-Lafeve, 2000). In addition, co-stimulation of the ADP P_2Y_{12} receptor and thromboxane A2 receptor are also required for platelet aggregation and secretion induced by low concentrations of collagen (Atkinson et al., 2001, Roger et al., 2004). ADP mediates its effect through two purinergic receptors coupled to G proteins, P_2Y_1 and P_2Y_{12}. As already stated, NADA inhibits platelet aggregation through at least pathway interfering/blocking the ADP receptor (P_2Y_{12}) (Chapter 3).

NADA was the only vanilloid that inhibited aggregation induced by collagen in SLE patients. The reason why NADA had an effect with collagen but not ADP is unclear, however it is interesting to speculate on possible reasons for this data. Although collagen receptors have not been investigated as possible targets for vanilloid action, NADA has been shown to produce an inhibitory effect on collagen-induced aggregation in healthy platelets (Almaghrabi et al., 2014). Therefore, NADA may

interfere with P_2Y_{12} ADP receptor (Chapter 3) and collagen receptor/s. The effect of NADA was more prominent with collagen-induced aggregation using SLE platelets because the P_2Y_{12} ADP receptor has been found to be down-regulated in SLE patients (Wang et al., 2004). Other possible explanations may be variations in receptor expression and function and/or alterations to intracellular signaling pathways in platelets from SLE patients' platelets compared to healthy platelets that changed NADA's molecular target.

Several limitations of this study need to be considered. Firstly, the small sample size might increase the risk of exaggerating treatment effects compared to larger sample size (Moher et al., 1998, Schulz et al., 1995). Secondly, the platelet count for aggregation experiments was standardised to 250 x 10^9/L in a PRP preparation and thus might not reflect the natural behaviors of platelets from SLE patients either with regard to platelet count or their interactions with other components of whole blood. As some patients have thrombocytopenia (Fayyaz et al., 2015, Jung et al., 2016), increasing the number of platelets might mask the actual effect of vanilloid-like agents. Finally, SLE patients were taking aspirin and/or other NSAIDs, and thus the effects of vanilloid-like agents on AA-induced aggregation could not be investigated.

Future direction could include: 1) increase the number of patients as it may show the actual effect of vanilloids and decrease the discrepancy and variability between the data, 2) use a whole blood aggregometry technique, which does not need platelet count standardisation and probably more accurately mimics the *in vivo* conditions, and 3) determine the effect of vanilloids on AA-induced aggregation by measuring TXB2 formation.

In conclusion, the current pilot study showed that NADA had a significant inhibitory effect on aggregation induced by collagen on platelets from SLE patients. However, unlike those from the healthy individual the plant-derived vanilloids, CAP and DHC, as well as the endovanilloid, OLDA, had no effect on ADP- or collagen-induced *in vitro* platelet aggregation of SLE platelets. Capsaicin, DHC and OLDA had no effect on SLE platelets either because of the disease itself and/or the medications. This is the first study to generate pilot data on the effect of plant-derived vanilloids, CAP and DHC, and the endovanilloids, OLDA and NADA, on SLE platelet aggregation. The data generated provides the basis to further investigate the inhibitory effects of NADA toward platelets from a larger number of SLE patients and other patients with high risk of thrombosis.

6. Chapter 6: Discussion

In this thesis the possible mechanisms by which vanilloid-like agents including, plant-derived vanilloids and endovanilloids/endocannabinoids, inhibit aggregation of human platelets have been investigated. The reasons for conducting these experiments were that these agents produced significant inhibitory effects on platelet aggregation induced by several platelet agonists, which is unlike most available conventional antiplatelet medications that usually act through a single target. Therefore, a clear understanding of how vanilloid-like agents act on platelets, i.e., through vanilloid receptors (e.g., TRPV1, CB1, CB2), other platelet receptors (e.g., P_2Y_1, P_2Y_{12}) and/or signaling pathways (e.g., AA pathway), may provide a basis to consider using these compounds, either alone or in combination, as therapeutic options as anti-aggregation agents. As antiplatelet medications have been shown to have a beneficial effect in the primary and secondary prevention of CVD, developing novel anti-platelet drugs with fewer side effects is a major focus of pharmaceutical companies.

Four main studies were conducted in this thesis; 1) investigations into the mechanism(s) of action of vanilloid-like agents on platelets using TRPV1, CB1 and CB2 receptors antagonist (Chapter 2), 2) measuring the end products of ADP receptor and AA pathway activation (Chapter 3), 3) determination of the effect of the combination of plant-derived vanilloids, CAP and DHC, on *in vitro* platelet aggregation and the AA pathway (Chapter 4), and 4) a pilot clinical study to investigate the effect of vanilloids on *in vitro* platelet aggregation in SLE patients (Chapter 5).

In neuronal tissue CAP, OLDA and NADA act through TRPV1, a non-selective cation channel with some preference for Ca^{2+} (Caterina et al., 1999, Zhong and Wang, 2008). The endovanilloids/endocannabinoids, OLDA and NADA, also have an affinity for CB receptors (Hu et al., 2009). Although the expression of CB1 and CB2 receptors has previously been demonstrated in platelets, only one study, using Western blotting, has shown that TRPV1 is present on platelets (Deusch et al., 2004, Harper et al., 2009). For the first time, I was able to confirm the presence of, and visualise TRPV1 channels on/in platelets using the highly sensitive technique of confocal microscopy, which allowed further investigations of whether these channels are involved in the vanilloids induced inhibition of platelet aggregation (Chapter 2).

Subsequently, the possible role of TRPV1 channels, and CB1 and CB2 receptors, on the inhibition of platelet aggregation produced by vanilloids and endovanilloids was investigated. Platelet aggregation was induced using three agonists, collagen, AA, and ADP, in combination with vanilloids and/or endovanilloids. To ensure that vanilloids, endovanilloids and receptor antagonists used in this study had no direct effect on platelets, platelet numbers were also determined after exposure to these agents and shown to have no effect on platelet count (Chapter 2).

The potent antagonists to TRPV1 channels, as well as the CB1 and CB2 receptors each had no effect on platelet aggregation. Blocking these receptors did not interfere with the inhibitory effects of OLDA and NADA on collagen-induced aggregation or CAP and NADA in AA-induced aggregation. However, blocking TRPV1 channels with SB452533 enhanced the inhibitory effect of OLDA in collagen-induced aggregation and CAP in AA-induced aggregation, which could be due to reduced

Ca^{2+} influx $[Ca^{2+}]_i$ and/or intracellular Ca^{2+} release from platelets (Clapham, 2003, Montell et al., 2002, Moran et al., 2004). In addition, blocking the TRPV1 receptor may decrease binding of CAP and OLDA to TRPV1, thus increase their bioavailability for other anti-aggregating activities. This could be investigated by performing functional /pharmacological studies, including Ca^{2+} flux, and radioligand binding. Moreover, the antiplatelet effect of CAP in AA-induced aggrgation was also enhanced in the presance of the CB2 antagonist that may result in increased cAMP formation (Rueda et al., 2000, Childers and Deadwyler, 1996). The inhibitory influence of CAP and OLDA on aggregation induced by ADP was therefore independent of CB1 and CB2 receptors (Chapter 2).

Despite the new data presented in this thesis the precise roles of TRPV1 channels in platelets remain unclear. These channels may be involved in the pro/inflammatory response of platelets, as TRPV1 expression is upregulated in different inflammatory diseases and platelets play a pivotal role in many inflammatory conditions (Chan et al., 2003, Yiangou et al., 2001, Hansson and Libby, 2006, Robbie and Libby, 2001). The results of the current study are in agreement with previous work that demonstrated that the CAP inhibition of canine platelet aggregation was not TRPV1 mediated (Mittelstadt et al., 2012). In contrast, another study demonstrated that TRPV1 channels are present in human platelets, but that CAP enhanced platelet aggregation in the absence of ADP or other platelet agonists (Harper et al., 2009). This effect led to increased intracellular Ca^{+2} through TRPV1, which was blocked in the presence of a TRPV1 antagonist (Harper et al., 2009). The conflicting data concerning the effect of CAP platelet aggregation might be due to differences in study design where the current study investigated the effect of CAP on exogenously

activated platelet aggregation, while in Harper et al. the direct effect of CAP on platelet aggregation was tested. Collectively, this data suggests that TRPV1 channels, and CB1 and CB2 receptors, are not directly involved in the anti-platelet actions of vanilloids and endovanilloids/endocannabinoids in humans.

As vanilloid-like agents produced prominent inhibitory effects on AA- and ADP-induced aggregation (Almaghrabi et al., 2014, Adams et al., 2009), the effects of these compounds on the end products of their respective signaling pathways were next investigated. Firstly, TXB2 concentration (an end product of AA pathway) was measured in the presence and absence of CAP, DHC, NADA and OLDA. Plant-derived vanilloids, CAP and possibly DHC, appeared to inhibit TXB2 formation in a concentration-dependent manner, which paralleled their effect on *in vitro* platelet aggregation (Chapter 3). My results are in agreement with Raghavendra and Naidu (2009) who showed that CAP inhibits cyclooxygenase1 (COX1) in the AA pathway, which may result in a dampening of platelet aggregation. In contrast, NADA did not show any effect on TXB2 levels, although it inhibited AA-induced platelet aggregation. NADA may interfere with the ADP receptor, P_2Y_{12}, as ADP release from platelet granules is a part of the amplification process of AA-induced aggregation. Moreover, OLDA had no effect on TXB2 levels, which was expected as OLDA has been previously shown to have no effect on AA-induced aggregation (Almaghrabi et al., 2014).

Secondly, the effect of CAP, DHC, OLDA and NADA on ADP-induced α- and dense-granules release via the P_2Y_1 receptor was investigated. Vanilloid-like agents do not appear to inhibit platelet aggregation through interfering/blocking the P_2Y_1

receptor, as PF4, β-TG or 5-HT release from ADP-activated platelets were not affected (Chapter 3). Hence, whether the inhibitory effects of plant-derived vanilloids and endovanilloids through the second ADP receptor (P_2Y_{12}) was determined by measuring levels of VASP phosphorylation. NADA, and possibly OLDA, appeared to inhibit platelet aggregation through interference with the P_2Y_{12} receptor, as VASP phosphorylation was significantly increased (Chapter 3). In contrast, CAP inhibited ADP-induced aggregation but had no effect on VASP phosphorylation status. This may be due to a high concentration of ADP used in the VASP ELISA kit (which was not supplied by the manufacturer), which was able to overcome any inhibitory effect of CAP. As expected, DHC did not show any effect on VASP phosphorylation level since it had no effect on ADP-induced aggregation. It can be concluded from this data that CAP and DHC may act at least partially through suppression of the AA pathway, and NADA and OLDA through interference to the P_2Y_{12} receptor, to inhibit platelet aggregation (Chapter 3). The knowledge of how vanilloids interact and/or target receptors and pathways in platelets is potentially beneficial, in that it might be possible to form a nutraceutical combination of both plant-derived vanilloids and endovanilloids that produce a higher antiplatelet effect. Finally, lacking of a clear understanding of mechanism(s) of action of vanilloids or (any drug) may lead to deleterious outcomes.

Platelet-derived microparticles (PMP) have been increasingly recognised as markers of platelet activation, which is associated with increased risk of CVD (Abrams et al., 1990, Morel et al., 2011a, Keuren et al., 2006, Azevedo et al., 2007, Hartopo et al., 2016). To my knowledge there are no previous studies that have investigated the influence of vanilloids on PMP formation/release. Therefore, the effects of vanilloids

on PMP generation/release induced by ADP and AA were determined. Circulating levels of CD41b/CD42a-positive PMP in the presence of CAP, DHC, OLDA and NADA were not significantly altered. In ADP-stimulated platelets, NADA and OLDA appeared to inhibit PMP release whereas with AA-stimulated platelets NADA slightly enhanced PMP formation. However, CAP and DHC enhanced PMP formation with both ADP and AA, in contrast to their inhibitory effect on platelet aggregation (Adams et al., 2009, Almaghrabi et al., 2014). Since platelet activation is associated with high levels of PMP release, the safety of using CAP and DHC as antiplatelet agents may be questionable, particularly by patients with hyperactive platelets, and warrants further investigation (Chapter 3). For example, use an *in vivo*/*ex vivo* model of CAP and DHC and their interactions with platelets may help determine their effects on PMP formation.

Plant-derived vanilloids, CAP and DHC, are the main capsaicinoids in *Capsicum* fruits (Bennett and Kirby, 1968, Kosuge and Furuta, 1970), and contribute ~60% and ~40%, respectively, to the total capsaicinoid concentration (Garces-Claver et al., 2006). In previous studies, I investigated the influence of CAP and DHC individually on platelet aggregation (Adams et al., 2009, Almaghrabi et al., 2014). Therefore, in the next set of experiments, the effect of a combination of CAP and DHC in the proportion they are usually present naturally, on AA-, ADP- and collagen-induced aggregation, platelet count and TXB2 formation, was investigated (Chapter 4). The combination of CAP and DHC had a greater inhibitory effect on *in vitro* AA-induced aggregation compared to their individual effects, suggesting these molecules may act synergistically. The combined concentration was lower than the individual CAP and DHC concentrations, but the combined effects were greater. In contrast, at the

concentrations tested, CAP and DHC individually, and in combination, had no significant effect on ADP- or collagen-induced aggregation (Chapter 4). CAP and DHC, and their combination, had no direct effect on platelet count. Moreover, the low CAP:DHC combination inhibited *in vitro* platelet aggregation through the AA pathway, as TXB2 formation decreased significantly in the presence of combined CAP:DHC. In comparison, the effect of 50 μM of CAP and DHC individually on TXB2 level was 10.7% and 4.6%, respectively (Chapter 3), while the combination of CAP and DHC (7.5:5 μM) was 4.4%, reflecting that low concentrations in combination are more effective in inhibiting platelet aggregation than higher concentrations of the individual vanilloid. On the contrary, low concentrations of individual CAP and DHC (12.5 μM) showed no effect on TXB2 formation (Chapter 4). These results suggest that CAP and DHC might inhibit different enzymes or act synergistically on one of the enzymes in AA pathway, to inhibit TXA2 formation and subsequently TXB2.

In addition to the anti-platelet effect of CAP:DHC combination (Chapter 4), it has been reported that individuals who regularly consume chilli have a lower incidence of peptic ulcers than those who eat chilli less frequently (Kang et al., 1995). Gastric injury and peptic ulcers are the most common side effects of aspirin (Singh and Triadafilopoulos, 1999, Derry and Loke, 2000, Ivey et al., 1980, Weil et al., 1995). Several studies have shown that low doses of CAP have a gastro-protective effect through increased mucosal secretion, which prevents gastric mucosal damage caused by indomethacin, ethanol and /or aspirin (Debreceni et al., 1999, Mozsik et al., 1999, Mozsik, 2014, Szabo et al., 2013, Yeoh et al., 1995). Although CAP inhibits *in vitro* platelet aggregation, a study showed that CAP does not have an additional inhibitory

effect in combination with aspirin, compared to aspirin alone, across a 24 hour period (Sandor et al., 2014). However, an endoscopic study has reported a lower gastric injury in volunteers who ingested chilli with aspirin (Yeoh et al., 1995). Given that combined CAP:DHC had a great antiplatelet effect, as well as the protective effect that natural chilli appears to have on the gastric mucosa (Yeoh et al., 1995, Kang et al., 1995), further work should investigate the combined effects of CAP and DHC (or chilli) and aspirin, against aspirin only, on platelet aggregation.

The effects of vanilloid-like agents have been tested in all previous studies using healthy platelets from different species, i.e., human, rabbits and canine (Almaghrabi et al., 2014, Mittelstadt et al., 2012, Hogaboam and Wallace, 1991, Harper et al., 2009, Adams et al., 2009, Raghavendra and Naidu, 2009). In this thesis however, the influence of these compounds on platelets from SLE patients who have a high tendency of thrombosis and atherosclerosis were determined, in a pilot study (Chapter 5) (Ward, 1999, Gladman and Urowitz, 1987). Plant-derived vanilloids, CAP and DHC, and the endovanilloid, OLDA, did not inhibit platelet aggregation induced by ADP and collagen. Only NADA inhibited collagen-induced *in vitro* platelet aggregation in a concentration-dependent manner, but no effect on ADP-induced aggregation was observed. The reason(s) for the differences between the effects of vanilloid-like agents on healthy and SLE patients' platelets are unclear. It may be that the pathway(s) by which vanilloid-like agents inhibit platelet aggregation in healthy platelets is impaired, blocked or over-stimulated by SLE itself, or affected by medication(s).

NADA is the only vanilloid that inhibited aggregation induced by ADP, AA and collagen using healthy platelets (Almaghrabi et al., 2014), as well as collagen-induced aggregation using platelets from SLE patients (Chapter 5). Furthermore, NADA demonstrated decreased PMP formation, at least with ADP-activated platelets (Chapter 3). In addition to NADA's antiplatelet effect it has also been shown to have anti-inflammatory properties and may play an important role in the immune system (Wilhelmsen et al., 2014, Sancho et al., 2004). Therefore, it would be interesting to further investigate the physiological interactions between NADA and platelets through *in vivo/ex vivo* models of disease, specifically in disorders of the cardiovascular system.

The main limitations of these studies were: first, there was a relatively small catchment of SLE patients or other patients with thrombophilia around Launceston. This was compounded by the absence of a rheumatology clinic at the Launceston General Hospital, which made it difficult to recruit a large number of patients. Further, the experimental conditions dictated that the blood samples had to be processed within four hours of collection to ensure the viability of platelets. Second, PRP was used to determine *in vitro* aggregation in response to various agents, which may not accurately reflect *in vivo* platelet function. Third, higher concentrations of the platelet agonists, ADP and AA, could be used in PMP experiments, but concentrations of all agonists and vanilloids were kept consistent across all experiments to allow comparison of the effect of vanilloids on platelets across different studies. Finally, there may be a variable response of platelets from the individual subject to agonists and/or vanilloids.

Future directions could include: 1) increasing the SLE cohort to investigate expression of TRPV1 in SLE platelets compared to the healthy individuals, 2) use of whole blood aggregometry e.g., Multiplate Analyser, and two or more platelet agonists together to determine the effects of vanilloids on platelet aggregation, which more closely mimic *in vivo* conditions, 3) use a higher concentration of agonist for PMP experiments, 4) investigate the role of fibrinogen receptor (GPIIb/IIIa) on the effect of vanilloids on platelet aggregation, 5) explore the potential role of TRPV1, CB1 and CB2 receptors in platelets, and 6) determine the *in vivo/ex vivo* effect of the CAP, DHC (or chilli) and aspirin, against aspirin only, on platelet aggregation using healthy individual and SLE patients platelets.

In conclusion, the results from this thesis confirmed the presence of TRPV1 channels on healthy platelets. Moreover, the inhibitory influence of plant-derived vanilloids, CAP and DHC, and endovanilloids/endocannabinoids, OLDA and NADA, on *in vitro* platelet aggregation are not TRPV1- or CB1- or CB2-mediated. However, blocking TRPV1 and CB2 receptors may enhance the anti-aggregating effects of OLDA and CAP. CAP and DHC appear to inhibit *in vitro* platelet aggregation by suppression of the AA-pathway, whereas NADA and OLDA do so by interfering with and/or blocking the ADP receptor P_2Y_{12}. However, none of the vanilloids appears to inhibit platelet aggregation by interfering with the P_2Y_1 receptor or have an effect on PMP formation/release. Interestingly, the combination of CAP and DHC act synergistically to inhibit aggregation induced by AA and TXB2 formation. In the pilot study using platelets from SLE patients only NADA produced an inhibitory effect on collagen-induced aggregation, whereas CAP, DHC and OLDA had no effect on ADP- or collagen-induced aggregation in the patient samples. Finally,

further studies are clearly warranted, especially considering the clinical potential of the chilli pepper in healthy and SLE patients, as additional therapeutic nutraceutical to reduce cardiovascular risk, and to decrease the side effect/s of conventional antiplatelet medication/s.

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Appendices

Appendix-1: Copies of conferences posters

Effect of Vanilloid-like Agents on Platelet Aggregation is Not Mediated Through Cannabinoid Receptors

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Background

Vanilloid-like agents, including capsaicin, the 'hot' principle in chilli, and the endocannabinoid/endovanilloid N-oleoyldopamine (OLDA), inhibit *in vitro* platelet aggregation. However, this effect is not through transient receptor potential vanilloid channel-1[1,2], a channel that mediate the action of vanilloids in other cells.

Endocannabinoid/endovanilloid compounds are produced by the body and bind to CB receptors that are present in many brain regions. Cannabinoid receptors (CB1, CB2) are present in platelets but their role is unclear. Knowing the mechanism of vanilloid in inhibiting platelet aggregation might provide a protection against cardiovascular disease.

Aim

To investigate whether the inhibitory effects of vanilloid-like agents is mediated through platelet CB1, CB2 receptors.

Method

1. Venous blood was collected from healthy participants in citrate tubes to obtain platelet -rich and platelet –poor plasma.
2. The effects of capsaicin and OLDA, on *in vitro* platelet aggregation, were determined in the absence and presence of CB1 (AM251) and CB2 (AM630) receptor antagonist (10 and 50 μM).
3. Platelet aggregation was induced by ADP (5 μM) (AggRAM platelet aggregometer, Helena Laboratories, Beaumont, USA).
4. Results for percent maximum aggregation (Fig. 1) were compared between blank (i.e., no vanilloid or antagonist) and test (i.e., 50 μM vanilloid ± 10 or 50 μM antagonist) using repeated measures ordinal logistic regression (Stata version 13, StataCorp, USA).

Results

Capsaicin and OLDA inhibited ADP-induced platelet aggregation significantly by 30.3% and 37.2%, respectively ($p < 0.05$) compare to ADP alone.

Blocking CB receptors using potent CB1 and CB2 receptor antagonist (10 and 50μM) did not affect the inhibitory action of capsaicin nor OLDA ($p > 0.05$) (Fig. 2).

Conclusion

The inhibitory effects of capsaicin and OLDA on platelets are not mediated through CB1 or CB2 receptors.

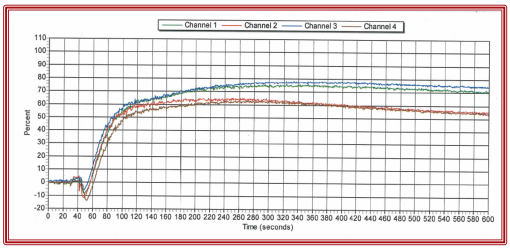

Fig. 1. The effect of vanilloids ± CB2 antagonist on platelet aggregation induced by 5μM ADP
5μM ADP
5μM ADP + 50μM CAP
5μM ADP + 50μM AM630
5μM ADP + 50μM CAP + 50μM AM630

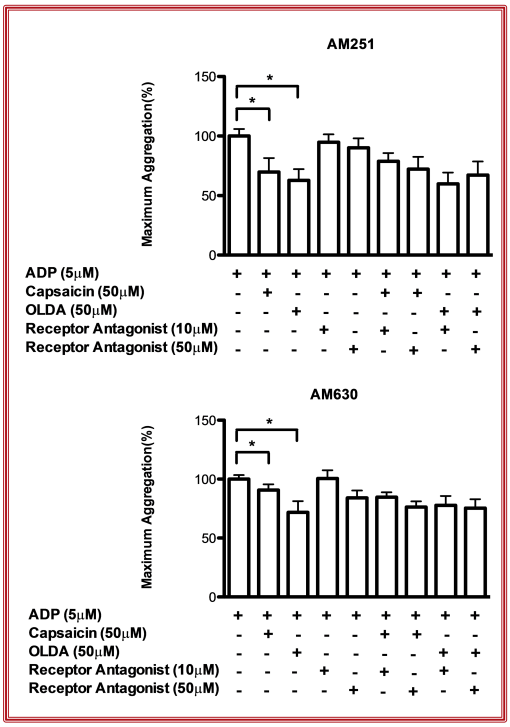

Fig. 2. Data show a maximum percent of aggregation induced by ADP. CB1 antagonist (AM251) and CB2 antagonist (AM630) do not affect the inhibition of platelet aggregation by OLDA and capsaicin. Results are the mean ± SEM of n=4. *p<0.05 compared to blank (i.e. 0 μM of vanilloid and antagonist)

Acknowledgments
Funding is provided by King Abdul Aziz University, Jeddah, Saudi Arabia and Clifford Craig Medical Research Trust, Launceston, Australia.

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2. Almaghrabi SY, Geraghty DP, Ahuja KD ,Adams MJ. Vanilloid-like agents inhibit aggregation of human platelets. Thromb Res 2014; 134:412-7.

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EFFECT OF VANILLOID-LIKE AGENTS ON PLATELET AGGREGATION IS NOT MEDIATED THROUGH TRANSIENT RECEPTOR POTENTIAL VANILLOID -1 OR CANNABINOID RECEPTORS

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Introduction/Background

Vanilloid-like agents such as the capsaicinoids [capsaicin (CAP) and dihydrocapsaicin (DHC)], as well as endocannabinoids/endovanilloids [N-oleoyldopamine (OLDA) and N-arachidonoyl-dopamine (NADA)], have been shown to inhibit *in vitro* platelet aggregation[1,2]. This inhibitory effect may provide protection against cardiovascular diseases.

Capsaicinoids and endocannabinoid/endovanilloids exhibit different affinities to transient receptor potential vanilloid-1 (TRPV1) and cannabinoid receptors (CB1, CB2) in neuronal tissue[3]. Platelets express TRPV1, CB1 and CB2 receptors, although their precise role(s) in platelet function are unclear[4,5].

Objectives/Study questions

1. To investigate the viability of platelets in the presence of capsaicinoids, endovanilloids and TRPV1, CB1 and CB2 receptor antagonists.
2. To determine whether inhibition of platelet aggregation by capsaicinoids and endovanilloids is mediated through platelet TRPV1, CB1 and/or CB2 receptors.

Methods

1. Platelet-rich and -poor plasma were obtained from venous blood of healthy subjects for both experiments.
2. A Sysmex 1000i analyzer (Roche Diagnostics) was used for platelet count experiments:
   - The effects of CAP, DHC, OLDA and NADA (3.125 and 50μM) and TRPV1 (SB452533), CB1 (AM251) and CB2 (AM630) antagonists (50 μM) on platelet count, compared to normal saline buffer pH 7.1 (buffer control), were determined, before and after adding these reagents, for two hours.
   - Data are presented as percent of platelet count normalised to platelet count at time = 0 min.
3. An AggRAM platelet aggregometer (Helena Laboratories) was used for aggregation experiments:
   - The effects of 50 μM CAP and NADA on aggregation induced by arachidonic acid (AA, 300 μg/mL), and of 50 μM OLDA and NADA induced by collagen (4 μg/ml), were determined in the absence and presence of SB452533, AM251 and AM630 (10, 50 μM).
   - Results for percent maximum aggregation were compared between blank (i.e., no vanilloid or antagonist) and test (i.e., 50 μM vanilloid ± 10 or 50 μM antagonist) using ordinal logistic regression (stata version 13, StataCorp, USA).

Results

1. Relative to buffer control, neither capsaicinoids/endovanilloids, nor antagonists, had an effect on platelet count after two hours of incubation (Fig. 1).
2. Collagen-induced aggregation (Fig. 2):
   - OLDA and NADA significantly inhibited platelet aggregation by 22.8% and 86.1%, respectively (Fig. 3C).
   - The inhibitory effect of OLDA was significantly enhanced by 50 μM SB452533 (39.4%), compared to OLDA alone (Fig. 3C).
   - The inhibitory effect of NADA was significantly enhanced by 50 μM AM630 (34.9%), compared to NADA alone (Fig. 3B).
3. AA-induced aggregation:
   - CAP and NADA inhibited platelet aggregation by 56.1% and 25.8%, respectively (Fig. 4C).
   - SB452533, AM251 and AM630 did not affect CAP- or NADA-induced inhibition of aggregation (Fig.4).

Implications/Conclusions

Capsaicinoids/endovanilloids and receptor antagonists had no effect on platelet viability. The inhibitory effects of CAP, OLDA and NADA on platelets are not mediated through TRPV1, CB1 or CB2 receptors. However, blocking TRPV1 and/or CB2 receptors may contribute to the action of capsaicinoids/endovanilloids.

Funding source(s)

Funding is provided by King Abdul Aziz University, Jeddah, Saudi Arabia and the Clifford Craig Medical Research Trust, Launceston, Australia.

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2. Almaghrabi SY et al (2014) Thromb Res, 134: 412-7
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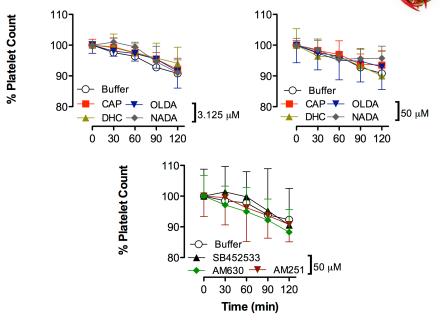

Fig. 1. CAP, OLDA, NADA, DHC, SB452533, AM251 and M630 have no effect on platelet count over a two hour incubation period. Data are presented as percent of platelet count (normalised to platelet with buffer only). Results are the mean ± SEM of 4 experiments.

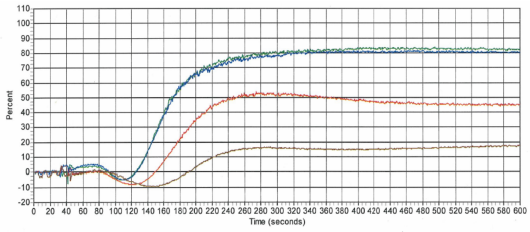

Fig. 2. The effect of vanilloids ± TRPV1 antagonist on platelet aggregation induced by 4μg/ml Collagen
Channel 1: 4μg/ml Collagen
Channel 2: 4μg/ml Collagen + 50μM OLDA
Channel 3: 4μg/ml Collagen + 50μM SB452533
Channel 4: 4μg/ml Collagen + 50μM OLDA + 50μM SB452533

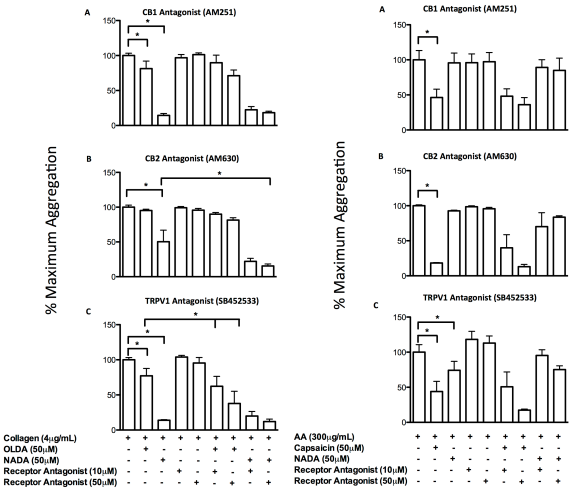

Fig. 3. Data show percent of maximum aggregation induced by collagen. CB1 (AM251) CB2 (AM630) and TRPV1 antagonists (SB-452533) do not affect the inhibition of platelet aggregation by OLDA and NADA. Results are the mean ± SEM of 4 experiments. *p<0.05 compared to blank (i.e. 0 μM of vanilloid and antagonist).

Fig. 4. Data show percent of maximum aggregation induced by AA. CB1 (AM251) CB2 (AM630) and TRPV1 antagonists (SB-452533) do not affect the inhibition of platelet aggregation by capsaicin and NADA. Results are the mean ± SEM of 4 experiments. *p<0.05 compared to blank (i.e. 0 μM of vanilloid and antagonist).

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Synergistic Effect of Capsaicin and Dihydrocapsaicin on *In Vitro* Platelet Aggregation

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[2]School of Veterinary and Life Sciences, Murdoch University, Perth, WA, Australia

Background

Pepper fruits (Capsicum) are one of the most consumed spices worldwide. These fruits contain capsaicinoids, which give them a characteristic pungent taste. Capsaicin (CAP) and dihydrocapsaicin (DHC) are the major capsaicinoids found in these fruits (1, 2) and are usually present in a 60:40 ratio, respectively (3). Individually, CAP and DHC have strong inhibitory effects on arachidonic acid (AA)-induced *in vitro* platelet aggregation, and to a lesser extent with ADP-induced platelet aggregation (4). In contrast, neither CAP nor DHC appear to have an inhibitory effect on collagen-induced aggregation (4).

It has been reported that low dose CAP causes a gastro-protective effect through increased mucosal secretion, and prevention of gastric mucosal damage caused by aspirin, indomethacin and ethanol (5-7). In addition, individuals who regularly consume chili have a lower incidence of peptic ulcers than those who eat chili less frequently (8).The inhibitory action of capsaicinoids on platelet aggregation could potentially be applied to the prevention and/or treatment of cardio- or cerebrovascular events, especially if they exhibit an anti-platelet and gastro-protective effects, as the gastric mucosal damage is one of the main side effects of conventional anti-platelet medications.

Objectives

1. To investigate the viability of platelets in the presence of CAP and DHC, and their combination.
2. To compare the effects of CAP and DHC alone, and in combination on *in vitro* platelet aggregation in the proportions they are present in pepper fruits.

Methods

1. Platelet-rich and -poor plasma were obtained from venous blood of healthy subjects.
2. A Sysmex 1000i analyzer (Roche Diagnostics) was used for platelet count experiments:
   - The effects of 12.5 µM CAP and DHC, and their combination (CAP:DHC, 7.5:5 µM) on platelet count, compared to normal saline buffer pH 7.1 (buffer control), were determined, before and after adding these reagents, for two hours.
   - Data are presented as percent of platelet count normalised to platelet count at time = 0 min.
3. An AggRAM platelet aggregometer (Helena Laboratories) was used for aggregation experiments:
   - The effects of 12.5 µM CAP and DHC, and their combination (CAP:DHC, 3.75:2.5 µM and 7.5:5 µM) on arachidonic acid (AA; 300 µg/mL), ADP (5 µM), and collagen (4 µg/mL) induced platelet aggregation, were determined.
   - Results for percent 'area under curve' were obtained (Fig. 1) and compared between control (i.e., agonist only) and test (i.e., agonist + capsaicinoid/s) using ANOVA/linear regression (Stata version 13, StataCorp, USA).

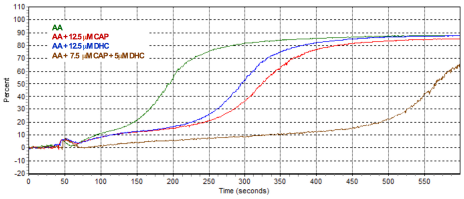

Fig. 1. Representative traces showing the effect of capsaicinoids on platelet aggregation induced by 300 µg/mL AA.

Results

1. Relative to buffer control, neither CAP nor DHC, nor their combination (CAP:DHC, 7.5:5 µM), had an effect on platelet count after two hours of incubation (Fig. 2).
2. Capsaicin and DHC (12.5 µM) individually inhibited AA-induced aggregation by 23.2% and 25.3%, respectively (both p<0.01), compared to control (Fig. 3A).
3. The combination of CAP and DHC produced further inhibition in AA-induced aggregation (CAP:DHC, 3.75:2.5 µM, 36.5%, p=0.01; CAP:DHC, 7.5:5 µM, 57.5%, p<0.001) compared to the control (Fig. 3A).
4. The inhibitory effect of combined CAP:DHC (7.5:5 µM) was larger than CAP and DHC individually by 34.3% and 32.2%, both p<0.05, respectively (Fig. 3A).
5. Neither CAP nor DHC, nor their combination, had a significant inhibitory effect on ADP- or collagen-induced aggregation (Fig. 3B & C).

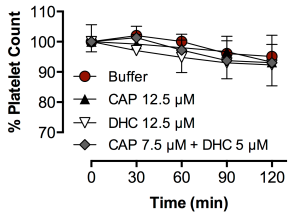

Fig.2. 12.5 µM of CAP and DHC, the combination of CAP:DHC (7.5:5 µM), have no effect on platelet count over 2 hours of incubation. Data are presented as per cent of platelet count (normalized to platelet count at time zero). Mean ± standard error of the mean (SEM); n = 4.

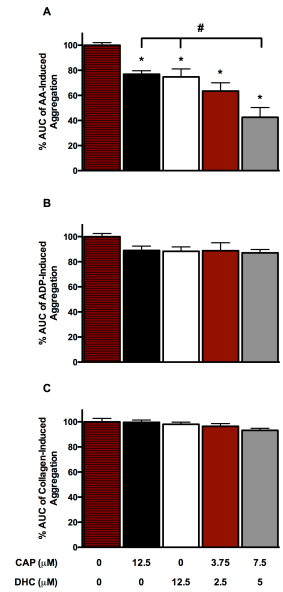

Fig. 3. The effects of Capsaicin (CAP), dihydrocapsaicin (DHC) and their combination on (A) arachidonic acid (AA)-, (B) ADP- and (C) collagen-induced platelet aggregation. Data are presented as percent of area under curve (%AUC) normalized to aggregation in the absence of capsaicinoid (control). Results are the mean ± SEM of 4 experiments. *significantly different from control; # significantly different from CAP (12.5 µM) or DHC (12.5 µM).

Conclusion

Combining CAP and DHC, in proportions found in pepper fruits, led to a greater inhibition of AA-induced platelet aggregation, compared to the individual effects of each capsaicinoid. Furthermore, this combined inhibitory effect was greater when using lower concentrations of each capsaicinoid, compared to their individual effects at higher concentrations. Moreover, this combination of capsaicinoids had no direct effect on the platelet count.

Funding source(s)
Funding is provided by King Abdul Aziz University, Jeddah, Saudi Arabia and the Clifford Craig Medical Research Trust, Launceston, Australia.

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Appendix-2: Published paper

Almaghrabi, SY, Geraghty, DP, Ahuja, KDK and Adams, MJ 2016. Inhibition of platelet aggregation by vanilloid-like agents is not mediated by transient receptor potential vanilloid-1 channels or cannabinoid receptors. *Clin Exp Pharmacol Physiol,* 43**,** 606-11.

Link to the manuscript:

http://onlinelibrary.wiley.com/doi/10.1111/1440-1681.12569/full

Adams, MJ, **Almaghrabi, SY**, Ahuja, KDK & Geraghty, DP 2013. Vanilloid-Like Agents: Potential Therapeutic Targeting of Platelets? *Drug Development Research,* 74**,** 450-459.

Link to the manuscript:

http://onlinelibrary.wiley.com/doi/10.1002/ddr.21102/abstract

Almaghrabi, SY, Geraghty, DP, Ahuja, KDK & Adams, MJ 2014. Vanilloid-like agents inhibit aggregation of human platelets. *Thromb Res,* 134**,** 412-7.

Link to the manuscript:

https://www.ncbi.nlm.nih.gov/pubmed/24953906

Appendix-3: Manual of VASP/P_2Y_{12} ELISA assay

CY-QUANT VASP/P2Y12

For the measurement of specific
platelet ADP receptor antagonists

Kit contents:

- 1 microtiter plate of Reagent 1 (96 Anti-VASP coated wells)
- 3 vials of Reagent 2a (PGE1)
- 3 vials of Reagent 2b (PGE1 + ADP)
- 1 vial of Reagent 3 (Lysis buffer)
- 1 vial of Reagent 4 (Washing solution)
- 1 vial of Reagent 5 (Dilution buffer)
- 1 vial of Reagent 6 (Anti-VASP-P peroxidase)
- 1 vial of Reagent 7 (TMB)
- 1 vial of Reagent 8 (Stop solution)
- 1 tool to extract wells from their strips

For In Vitro Diagnostic Use

Ref. 7502

CE

1- INTRODUCTION

CY-QUANT VASP/P2Y12 is an enzyme-linked immunosorbent assay (ELISA) procedure for the determination of serine 239-phosphorylated VASP (VASP-P) in platelets from fresh human whole blood.

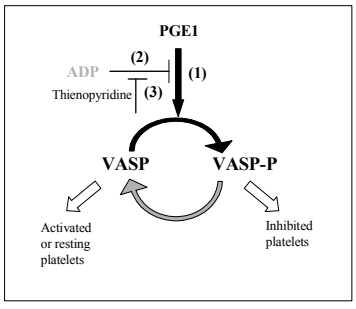
CY-QUANT VASP/P2Y12 kit is dedicated to the measurement of specific platelet ADP receptor (P2Y12) antagonists.

VASP (Vasodilator Stimulated Phosphoprotein) is an intracellular platelet protein which is unphosphorylated at basal state.

Prostaglandin E1 (PGE1) induces phosphorylation of VASP **(1)** whereas the binding of adenosine diphosphate (ADP) to P2Y12 receptors **(2)** leads to dephosphorylation of VASP. Under test conditions, in the concomitant addition of ADP and PGE1, the effect of ADP dominates and leads to VASP dephosphorylation, unless the P2Y12 receptor is efficiently blocked by antiplatelet drugs targeting this receptor (such as thienopyridines). Thus, the level of VASP phosphorylation in this condition reflects the level of P2Y12 receptor inhibition.

Inter-individual variability and resistance to antiplatelet drugs have been widely described [a,b]. The effect of thienopyridines **(3)** can be demonstrated with **CY-QUANT VASP/P2Y12** by the persistence of VASP phosphorylation induced by PGE1 despite simultaneous addition of ADP.

CY-QUANT VASP/P2Y12 may also be used to evaluate *in vitro* effects of P2Y12 receptor antagonists.



2- TEST PRINCIPLE

After a first step of parallel whole blood sample activation with PGE1 and PGE1+ADP (Reagent 2a and 2b), platelets from the sample are lysed (Reagent 3), allowing released VASP to be captured by an anti-human VASP antibody which is coated in the microtiter plate (Reagent 1). Then, a peroxidase-coupled anti-human VASP-P antibody (Reagent 6) binds to phosphorylated serine 239 antigenic determinant of VASP. The bound enzyme peroxidase is then revealed by its activity on TMB substrate (Reagent 7) over a predetermined time. After stopping the reaction (Reagent 8), absorbance at 450 nm is directly related to the concentration of VASP-P contained in the sample.

A platelet reactivity index (PRI) is calculated using optical density (OD_{450nm}) in the presence of PGE1 alone [*PGE1*] or PGE1 and ADP simultaneously [*PGE1+ ADP*].

3- REAGENTS

- **Reagent 1:** 96-wells microtiter plate composed of 12 breakable strips of 8 wells coated with mouse anti-human VASP MAb, in a re-sealable pouch.
- **Reagent 2a:** vial, lyophilized PGE1.
- **Reagent 2b:** vial, lyophilized PGE1 + ADP.
- **Reagent 3:** vial, 15 mL, lysis buffer.
- **Reagent 4:** vial, 50 mL, 20 fold-concentrated washing solution.
- **Reagent 5:** vial, 50 mL, dilution buffer.
- **Reagent 6:** vial, 1.6 mL, 20 fold-concentrated specific mouse anti-human VASP-P MAb coupled with peroxidase.
- **Reagent 7:** vial, 25 mL, TMB (tetramethylbenzidine).
- **Reagent 8:** vial, 15 mL, stop solution.

4- MATERIAL REQUIRED BUT NOT PROVIDED

- Deionized or distilled water, equilibrated at room temperature and preferably sterile.
- Timer.
- Multi-channel pipettes, pipettes with disposable tips.
- ELISA plate reader set at 450 nm.
- Vortex.
- Absorbent paper.

5- WARNING

- Follow the conventional laboratory practices.
- Follow the appropriate regulation for waste disposal.
- Blood must be considered as potentially infectious.
- Reagent 5:
  R43: May cause sensitization by skin contact.
  S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
  S28: After contact with skin, wash immediately with plenty of water.
  S36/37/39: Wear suitable protective clothing, gloves and eye/face protection.

6- REAGENTS PREPARATION AND STORAGE

Notes:

- When stored at 2-8° C, unopened kits and contents remain stable until the printed expiration date.
- Before use, all reagents must be equilibrated at room temperature (RT, 18-25 °C) for at least 30 minutes.

• **Reagent 1**

Ready-to-use. After first use, immediately replace unused strips and wells in the re-sealable pouch with the desiccant and store at 2-8°C.
Stability after opening: 2 months at 2-8°C, when free of contamination.

• **Reagents 2a and 2b**

Reconstitute each vial with **900 µL** of deionized or distilled water and homogenize the content using a vortex for 5 seconds.
Stability after reconstitution: 1 month at 2-8°C, when free of contamination.

• **Reagents 3, 5 and 8**

Ready-to-use. Stability after opening: 2 months at 2-8°C, when free of contamination.

• **Reagent 4**

Stability after opening: 2 months at 2-8°C, when free of contamination.
Before use, dilute the reagent **1:20** with deionized or distilled water.
For one well, dilute 100 µL of **Reagent 4** with 1 900 µL of deionized or distilled water.
Stability after dilution: 15 days at 2-8°C, when free of contamination.
Note: The presence of crystals does not affect the quality of the reagent. If necessary, warm at 37°C until all crystals have dissolved. Then, homogenize and equilibrate at RT.

• **Reagent 6**

Stability after opening: 2 months at 2-8°C, when free of contamination.
Before use, dilute the reagent **1:20** with **Reagent 5**.
For one well, dilute 15 µL of **Reagent 6** with 285 µL of **Reagent 5**.
Stability after dilution: 1 hour at RT.
Note: Since the vial is filled to capacity, pipette carefully to avoid reagent overflow.

• **Reagent 7**

Ready-to-use. Stability after opening: 2 months at 2-8°C, when free of contamination.
Note: Avoid exposure to light, heat and contamination with metal ions or peroxidase.

7- SPECIMEN COLLECTION AND STORAGE

- Draw venous whole blood into a **0.109 M trisodium citrate** collection tube, according to manufacturer's instructions.
- Maintain platelet integrity. Avoid platelet activation during the collection procedure (shaking, heat shock).
- Blood collection tube must be filled to capacity, stored at RT and unopened before the test.
- Samples must be analyzed within **24 hours** from collection.

8- PROCEDURE

We recommend to test a normal sample in parallel of each series, to serve as a control.

Notes:

- The washing steps can be performed either with an automated plate-washing equipment or manually with a multi-channel pipette.
- During manual washing, first empty all wells by flicking off the liquid into an appropriate container and blot the plate on a clean absorbent paper. Then, fill each well with 300 µL of diluted Reagent 4, flick off the washing solution, and blot the plate on a clean adsorbent paper.
- The number of washing steps must be scrupulously respected.
- Do not leave the wells dry at any time.
- Do not expose the strips to strong light.
- Check the absence of bubbles in the wells before OD measurement.

8.1- OPERATING PROCEDURE

At each step, identical incubation time must be carefully respected for each well.
Distribute the samples to be tested and the blank in duplicate; one duplicate blank is enough for a series of samples.
Pipette directly into precoated wells (Reagent 1):

		Well **PGE1**	Well **PGE1 + ADP**	Well **Blank**
ANTIGEN CAPTURE	Reagent:	**2a**: 40 µL	**2b**: 40 µL	**5**: 180 µL
	Whole blood sample:	40 µL	40 µL	—
	Thoroughly mix the contents of each well by pipetting up and down 8-10 times			—
	Cover the wells and incubate **10 minutes** at RT			—
	Reagent:	**3**: 100 µL	**3**: 100 µL	—
	Thoroughly mix the contents of each well by pipetting up and down 8-10 times			
	Cover the wells and incubate **30 minutes** at RT			
Wash all wells 3 times with 300 µL of diluted Reagent **4**, then add immediately:				
CONJUGATE IMMOBILIZATION	Diluted Reagent **6**:	200 µL	200 µL	200 µL
	Cover the wells and incubate **30 minutes** at RT			
Wash all wells 3 times with 300 µL of diluted Reagent **4**, then add immediately:				
COLOR DEVELOPMENT	Reagent **7**:	200 µL	200 µL	200 µL
	Incubate **5 minutes** at RT, and then add:			
	Reagent **8**:	100 µL	100 µL	100 µL
	Thoroughly homogenize the contents of each well			
OD PLATE MEASUREMENT	Measure the absorbance at **450 nm** up to **4 hours** at RT after stopping the reaction			

8.2- PLATELET REACTIVITY INDEX (PRI) CALCULATION

A platelet reactivity index (PRI) is calculated using optical density (OD_{450nm}) in the presence of PGE1 alone [*PGE1*] or PGE1 and ADP simultaneously [*PGE1+ ADP*], according to the following formula:

$$PRI\ (\%) = \frac{OD_{450nm}[PGE1] - OD_{450nm}[PGE1 + ADP]}{OD_{450nm}[PGE1] - OD_{450nm}[Blank]} \times 100$$

Note: Each laboratory must establish its own interpretation values depending upon the P2Y12 antagonist to evaluate.

In order to measure the efficiency of a P2Y12 antagonist, apply the following recommendations:

1- Determine the basal PRI range (mean ± 2 standard deviations) on a group of patients relevant of the disease of interest and not receiving the P2Y12 antagonist to evaluate. As a guide, the PRI of untreated healthy donors (n=32) is ranging from 89% to 99% (data from external study).
2- Determine the basal PRI value of the patient to be tested before the treatment (PRI_0) and confirm that this value is included in the pre-established basal PRI range. Otherwise, refer to the Limitations paragraph (§10) and repeat the test if necessary.
3- Determine the PRI value at a time point T (PRI_T) according to the pharmacodynamic properties of the P2Y12 antagonist evaluated. A PRI_T value which is still included in the basal PRI range signifies that the patient has not responded to the drug.

In summary, a low PRI corresponds to a good responder patient while a high PRI stands either for a healthy subject or a bad responder patient. The lower the PRI, the higher P2Y12 receptor inhibition.

9- PERFORMANCES

Repeatability:

Two samples presenting different levels of PRI are tested 8 times with the same kit:

Sample	Sample 1	Sample 2
n	8	8
$\bar{X}$ (PRI %)	43,69%	97,85%
SD	2,02	0,57
CV	4,6%	0,6%

Working range:

The working range for this method is from 0 up to 100 % of PRI.

Correlation to PLT VASP/P2Y12 (BioCytex ref. 7014, CE):

CY-QUANT VASP/P2Y12 test is strongly correlated with the flow cytometric PLT VASP/P2Y12 test: n = 96; r = 0.95; p < 0.001.

Interferences:

- Platelet count: on samples from 50,000 up to 375,000 platelets/µL, the platelet count has no significant interference on CY-QUANT VASP/P2Y12 assay.

- Red blood cell count: on non treated samples from 1 x 10^6 up to 5,8 x 10^6 red blood cells/µL, the red blood cell count has no significant interference on CY-QUANT VASP/P2Y12 assay.

- Aspirin and anti GpIIb/IIIa drugs have no significant interference on CY-QUANT VASP/P2Y12 assay since VASP biomarker is specific from the P2Y12 signaling pathway.

10- LIMITATIONS

CY-QUANT VASP/P2Y12 kit cannot be used on activated and/or hemolyzed blood samples.

11- LIABILITY

The *in vitro* diagnostic use is only valid within the strict application of the package insert. Any modification of the protocol may influence the result of the test.
Never switch or mix vials originating from different kits.
In cases where these recommendations are not strictly respected, no contestation or replacement of the product will be accepted.

12- REFERENCES

(a) Gurbel PA. *et al.* (2007) *Thromb Research* 120:311-321.
(b) Angiollilo D. *et al.* (2007) *J Am Coll Cardiol* 49:1505-1516.
(c) Barragan P. *et al.* (2010) *Thromb. Haemost* 104(2): 410-11.
(d) Jakubowski J.A. *et al.* (2012) *Thromb. Haemost* 107: 388-395.
(e) Abtan J. *et al.* (2013) *Thromb Haemost* 110(5):1055-64.

13- NOTICE TO PURCHASER

The **CY-QUANT VASP/P2Y12** kit is covered by patent WO 99/24473.

14- SYMBOLS

REF	*Catalogue number*		*Use By*
IVD	*In Vitro Diagnostic Medical Device*	Σ	*Contains sufficient for "n" tests*
	Temperature limitation	LOT	*Batch code*

BIOCYTEX
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Version March 2014

Appendix-4: Manual of 5-HT ELISA assay

CEA808Ge 96 Tests
Enzyme-linked Immunosorbent Assay Kit
For 5-Hydroxytryptamine (5-HT)
Organism Species: General
Instruction manual

FOR IN VITRO AND RESEARCH USE ONLY
NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

11th Edition (Revised in July, 2013)

[INTENDED USE]

The kit is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of 5-HT in serum and plasma.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
TMB Substrate	1×9mL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Microplate reader with 450 ± 10nm filter.
2. Precision single or multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution

[STORAGE OF THE KITS]

1. **For unopened kit:** All the reagents should be kept according to the labels on vials. The **Standard**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20ºC upon receipt while the others should be at 4 ºC.
2. **For opened kit:** When the kit is opened, the remaining reagents still need to be stored according to the above storage condition. Besides, please return the unused wells to the foil pouch containing the desiccant pack, and reseal along entire edge of zip-seal.

Note:

It is highly recommended to use the remaining reagents within 1 month provided this is within the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable until this expiration date.

[SAMPLE COLLECTION AND STORAGE]

Serum - Allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Assay immediately or store samples in aliquot at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

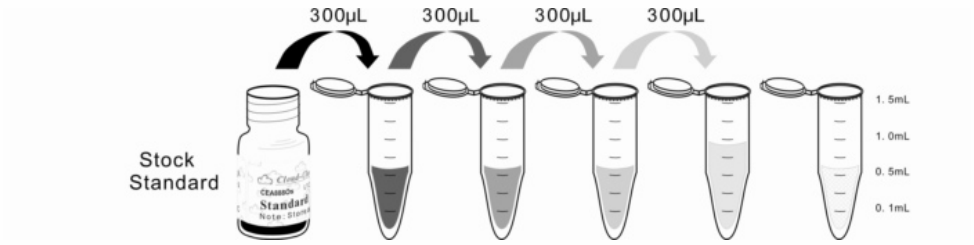
Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen should not be detected.
3. When performing the assay, bring samples to room temperature.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18-25°C) before use.
2. **Standard -** Reconstitute the **Standard** with 0.5mL of **Standard Diluent,** kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 900ng/mL. Please prepare 5 tubes containing 0.6mL Standard Diluent and produce a triple dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 5 points of diluted standard such as 900ng/mL, 300ng/mL, 100ng/mL, 33.33ng/mL, 11.11ng/mL, and the last EP tubes with **Standard Diluent** is the blank as 0ng/mL.



Tube	1	2	3	4	5	6
ng/mL	900	300	100	33.33	11.11	0

3. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with **Assay Diluent A** and **B,** respectively (1:100).

4. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
5. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standard within 15 minutes before assay. Please do not dissolve the reagents at 37°C directly.
3. Detection Reagent A and B are sticky solutions, therefore, slowly pipette them to reduce the volume errors.
4. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μL for once pipetting.
5. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.
6. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
7. Contaminated water or container for reagent preparation will influence the detection result.

[SAMPLE PREPARATION]

1. Cloud-Clone Corp. is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments. Sample should be diluted by 0.01mol/L PBS(PH=7.0-7.2).
3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
5. Due to the possibility of mismatching between antigen from other origin and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
6. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernatant may not be detected by the kit.
7. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 5 wells for standard points, 1 well for blank. Add 50μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells, respectively. And then add 50μL of Detection Reagent A to each well immediately. Shake the plate gently (using a microplate shaker is recommended). Cover with a Plate sealer. Incubate for 1 hour at 37°C. Detection Reagent A may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.

2. Aspirate the solution and wash with 350μL of 1X Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
3. Add 100μL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
4. Repeat the aspiration/wash process for total 5 times as conducted in step 2.
5. Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
6. Add 50μL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
7. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20°C.
2. **Samples or reagents addition：Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate **should not exceed 10 minutes.** This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes)**,** if the color is too deep, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Please protect it from light.
7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

[TEST PRINCIPLE]

This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to 5-HT has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled 5-HT and unlabeled 5-HT (Standards or samples) with the pre-coated antibody specific to 5-HT. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of 5-HT in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of 5-HT in the sample.

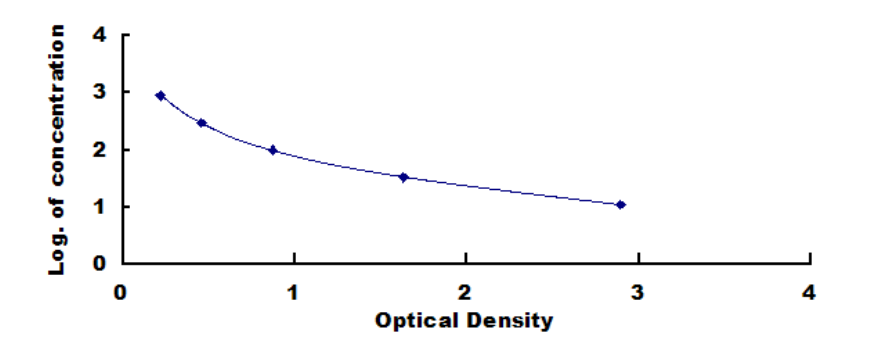
[CALCULATION OF RESULTS]

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between 5-HT concentration in the sample and the assay signal intensity.

Average the duplicate readings for each standard, control, and samples. Create a standard curve on log-log or semi-log graph paper, with the log of 5-HT concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis. Using some plot software, for instance, curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the log of concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. The O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Typical standard curve below is provided for reference only.



Typical Standard Curve for 5-HT ELISA.

[DETECTION RANGE]

11.11-900ng/mL. The standard curve concentrations used for the ELISA's were 900ng/mL, 300ng/mL, 100ng/mL, 33.33ng/mL, 11.11ng/mL.

[SENSITIVITY]

The minimum detectable dose of 5-HT is typically less than 3.92ng/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by subtracting two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

[SPECIFICITY]

This assay has high sensitivity and excellent specificity for detection of 5-HT.

No significant cross-reactivity or interference between 5-HT and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between 5-HT and all the analogues, therefore, cross reaction may still exist.

[RECOVERY]

Matrices listed below were spiked with certain level of 5-HT and the recovery rates were calculated by comparing the measured value to the expected amount of 5-HT in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=5)	96-105	101
EDTA plasma(n=5)	86-98	93
heparin plasma(n=5)	80-101	89

[LINEARITY]

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of 5-HT and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1：2	1：4	1：8	1：16
serum(n=5)	87-97%	79-104%	98-105%	92-102%
EDTA plasma(n=5)	93-101%	82-91%	78-90%	80-96%
heparin plasma(n=5)	89-103%	95-105%	85-101%	87-104%

[PRECISION]

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level 5-HT were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level 5-HT were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%

[STABILITY]

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage condition.

To minimize extra influence on the performance, operation procedures and lab conditions, especially room

temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

[ASSAY PROCEDURE SUMMARY]

1. Prepare all reagents, samples and standards;
2. Add 50μL standard or sample to each well.
   And then add 50μL prepared Detection Reagent A immediately.
   Shake and mix. Incubate 1 hour at 37°C;
3. Aspirate and wash 3 times;
4. Add 100μL prepared Detection Reagent B. Incubate 30 minutes at 37°C;
5. Aspirate and wash 5 times;
6. Add 90μL Substrate Solution. Incubate 15-25 minutes at 37°C;
7. Add 50μL Stop Solution. Read at 450 nm immediately.

[IMPORTANT NOTE]

1. Limited by the current condition and scientific technology, we can't completely conduct the comprehensive identification and analysis on the raw material provided by suppliers. So there might be some qualitative and technical risks to use the kit.
2. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
3. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for information.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
5. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.
6. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
7. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. or greater at 450 ± 10nm wavelength is acceptable for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment. For more information, please refer to the operation video (http://www.cloud-clone.us/homepage/operate.htm).
8. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before assay for each batch is recommended.
9. Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
10. Kits from different manufacturers with the same item might produce different results, since we haven't compared our products with other manufacturers.
11. The instruction manual also suits for the kit of 48T, but all reagents of 48T kit are reduced by half.

[PRECAUTION]

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

[TROUBLE SHOOTING]

Problem	Possible Source	Correction Action
Poor Standard Curve	Improper standard curve preparation	Ensure accurate operation of the dilution
	Incomplete washing and aspiration	Adequate washing and adequate aspiration
	Inaccurate Pipetting	Check and Calibrate pipettes
Poor Precision	Incomplete washing of wells	Ensure sufficient washing
	Inadequate mixing and aspiration reagents	Adequate aspiration and mixing reagents
	Reused pipette tips, containers and sealers	Change and use new pipette tips, containers and sealers
	Inaccurate Pipetting	Check and Calibrate pipettes
Low O.D Values	Inadequate reagent volumes added to wells	Calibrate pipettes and Add adequate reagents
	Incorrect incubation times	Ensure sufficient incubation times
	Incorrect incubation temperature	Reagents balanced to room temperature
	Conjugate or substrate reagent failure	Mix conjugate &substrate,color should develop immediately
	No stop solution added	Follow the assay protocol in the kit manual
	Read beyond suggested reading time	Read within the time recommended in the manual
Sample Values	Improper Sample Storage	Store the sample properly and use the fresh sample
	Improper sample collection and preparation	Take proper sample collection and preparation method
	Low quantity of analyte in samples	Use new sample and repeat assay

Appendix-5: Manual of PF4 ELISA assay

SEA172Hu 96 Tests
Enzyme-linked Immunosorbent Assay Kit
For Platelet Factor 4 (PF4)
Organism Species: Homo sapiens (Human)
Instruction manual

FOR IN VITRO AND RESEARCH USE ONLY
NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

11th Edition (Revised in July, 2013)

[INTENDED USE]

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of PF4 in human serum, platelet-poor plasma and cell culture supernates.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
TMB Substrate	1×9mL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Microplate reader with 450 ± 10nm filter.
2. Precision single or multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution

[STORAGE OF THE KITS]

1. **For unopened kit:** All the reagents should be kept according to the labels on vials. The **Standard**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20ºC upon receipt while the others should be at 4 ºC.
2. **For opened kit:** When the kit is opened, the remaining reagents still need to be stored according to the above storage condition. Besides, please return the unused wells to the foil pouch containing the desiccant pack, and reseal along entire edge of zip-seal.

Note:

It is highly recommended to use the remaining reagents within 1 month provided this is within the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable until this expiration date.

[SAMPLE COLLECTION AND STORAGE]

Serum - Allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Assay immediately or store samples in aliquot at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

Platelet-Poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge samples at 4°C for 20 minutes at 2,000×g within 30 minutes of collection. Centrifuge the separated plasma at 4°C for 10 minutes at 10,000×g. Assay immediately or store samples in aliquot at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

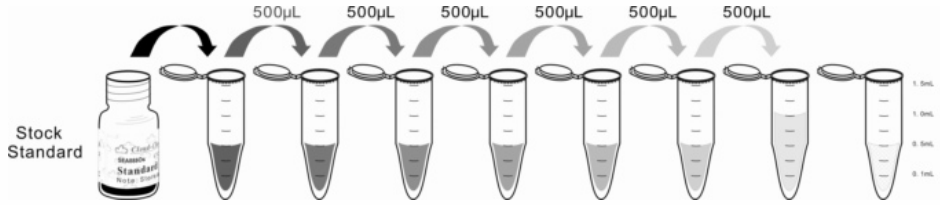
Cell culture supernates - Centrifuge samples for 20 minutes at 1000×g. Remove particulates and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen should not be detected.
3. When performing the assay, bring samples to room temperature.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18-25°C) before use.
2. **Standard -** Reconstitute the **Standard** with 1.0mL of **Standard Diluent,** kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 400pg/mL. Please firstly dilute the stock solution to 100pg/mL and the diluted standard serves as the highest standard (100pg/mL). Then prepare 7 tubes containing 0.5mL Standard Diluent and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 100pg/mL, 50pg/mL, 25pg/mL, 12.5pg/mL, 6.25pg/mL, 3.12pg/mL, 1.56pg/mL, and the last EP tubes with **Standard Diluent** is the blank as 0pg/mL.



Tube	1	2	3	4	5	6	7	8	9
pg/mL	400	100	50	25	12.5	6.25	3.12	1.56	0

3. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with **Assay Diluent A** and **B,** respectively (1:100).

4. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
5. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standard within 15 minutes before assay. Please do not dissolve the reagents at 37°C directly.
3. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μL for once pipetting.
4. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
6. Contaminated water or container for reagent preparation will influence the detection result.

[SAMPLE PREPARATION]

1. Cloud-Clone Corp. is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. Serum/plasma samples require about a 2,000 fold dilution. For example, to prepare a 1:2,000 dilution of sample, transfer 20μL of sample to 380μL PBS. This yields a 1:20 dilution. Next, dilute the 1:20 sample by transferring 10μL to 990μL PBS. You now have a 1:2,000 dilution of your sample. Mix thoroughly at each stage. Sample should be diluted by 0.01mol/L PBS(PH=7.0-7.2).
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
6. Due to the possibility of mismatching between antigen from other origin and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
7. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernatant may not be detected by the kit.
8. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank.
   Add 100μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.

2. Remove the liquid of each well, don't wash.
3. Add 100μL of **Detection Reagent A** working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.
4. Aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100μL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
6. Repeat the aspiration/wash process for total 5 times as conducted in step 4.
7. Add 90μL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
8. Add 50μL of **Stop Solution** to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20°C.
2. **Samples or reagents addition：Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes)**,** if the color is too deep, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Please protect it from light.
7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

[TEST PRINCIPLE]

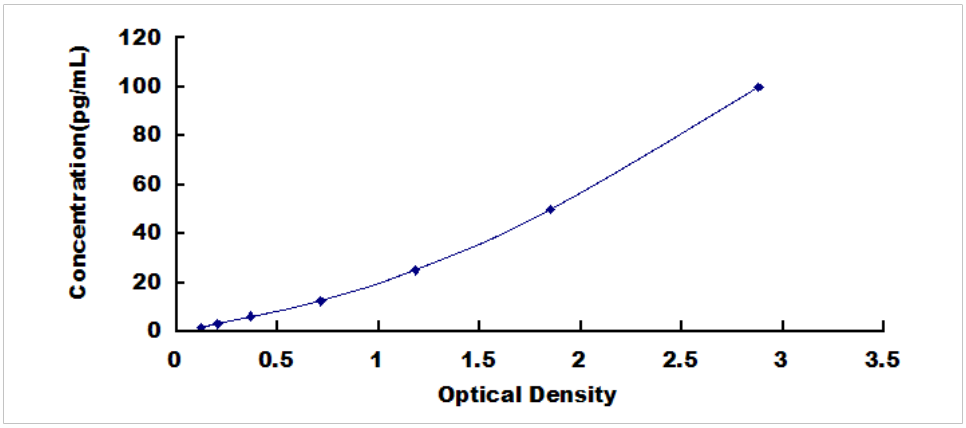
The microtiter plate provided in this kit has been pre-coated with an antibody specific to PF4. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to PF4. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain PF4, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of PF4 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

[CALCULATION OF RESULTS]

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D. and concentration for each standard and draw a best fit curve through the points on the graph or create a standard curve on log-log graph paper with PF4 concentration on the y-axis and absorbance on the x-axis. Using some plot software, for instance, curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the known concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. However, the O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), plotting log of the data to establish standard curve for each test is recommended. Typical standard curve below is provided for reference only.



Typical Standard Curve for PF4, Human ELISA.

[DETECTION RANGE]

1.56 -100pg/mL. The standard curve concentrations used for the ELISA's were 100pg/mL, 50pg/mL, 25pg/mL, 12.5pg/mL, 6.25pg/mL, 3.12pg/mL, 1.56pg/mL.

[SENSITIVITY]

The minimum detectable dose of PF4 is typically less than 0.68pg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

[SPECIFICITY]

This assay has high sensitivity and excellent specificity for detection of PF4.

No significant cross-reactivity or interference between PF4 and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between PF4 and all the analogues, therefore, cross reaction may still exist.

[RECOVERY]

Matrices listed below were spiked with certain level of recombinant PF4 and the recovery rates were calculated by comparing the measured value to the expected amount of PF4 in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=5)	85-98	92
EDTA plasma(n=5)	88-101	95
heparin plasma(n=5)	91-105	99

[LINEARITY]

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of PF4 and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1：2	1：4	1：8	1：16
serum(n=5)	89-98%	86-104%	80-98%	90-107%
EDTA plasma(n=5)	78-92%	80-93%	92-103%	88-99%
heparin plasma(n=5)	96-103%	85-99%	90-106%	81-95%

[PRECISION]

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level PF4 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level PF4 were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%

[STABILITY]

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage condition.

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

[ASSAY PROCEDURE SUMMARY]

1. Prepare all reagents, samples and standards;
2. Add 100μL standard or sample to each well. Incubate 2 hours at 37ºC;
3. Aspirate and add 100μL prepared Detection Reagent A. Incubate 1 hour at 37ºC;
4. Aspirate and wash 3 times;
5. Add 100μL prepared Detection Reagent B. Incubate 30 minutes at 37ºC;
6. Aspirate and wash 5 times;
7. Add 90μL Substrate Solution. Incubate 15-25 minutes at 37ºC;
8. Add 50μL Stop Solution. Read at 450nm immediately.

[IMPORTANT NOTE]

1. Limited by the current condition and scientific technology, we can't completely conduct the comprehensive identification and analysis on the raw material provided by suppliers. So there might be some qualitative and technical risks to use the kit.
2. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
3. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for information.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
5. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.
6. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
7. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. or greater at 450 ± 10nm wavelength is acceptable for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment. For more information, please refer to the operation video (http://www.cloud-clone.us/homepage/operate.htm).
8. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before assay for each batch is recommended.

9. Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
10. Kits from different manufacturers with the same item might produce different results, since we haven't compared our products with other manufacturers.
11. The instruction manual also suits for the kit of 48T, but all reagents of 48T kit are reduced by half.

[PRECAUTION]

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

[TROUBLE SHOOTING]

Problem	Possible Source	Correction Action
Poor Standard Curve	Improper standard curve preparation	Ensure accurate operation of the dilution
	Incomplete washing and aspiration	Adequate washing and adequate aspiration
	Inaccurate Pipetting	Check and Calibrate pipettes
Poor Precision	Incomplete washing of wells	Ensure sufficient washing
	Inadequate mixing and aspiration reagents	Adequate aspiration and mixing reagents
	Reused pipette tips, containers and sealers	Change and use new pipette tips, containers and sealers
	Inaccurate Pipetting	Check and Calibrate pipettes
Low O.D Values	Inadequate reagent volumes added to wells	Calibrate pipettes and Add adequate reagents
	Incorrect incubation times	Ensure sufficient incubation times
	Incorrect incubation temperature	Reagents balanced to room temperature
	Conjugate or substrate reagent failure	Mix conjugate &substrate,color should develop immediately
	No stop solution added	Follow the assay protocol in the kit manual
	Read beyond suggested reading time	Read within the time recommended in the manual
Sample Values	Improper Sample Storage	Store the sample properly and use the fresh sample
	Improper sample collection and preparation	Take proper sample collection and preparation method
	Low quantity of analyte in samples	Use new sample and repeat assay

Appendix-6: Manual of β-TG ELISA assay

SEA370Hu 96 Tests
Enzyme-linked Immunosorbent Assay Kit
For Beta-Thromboglobulin (bTG)
Organism Species: Homo sapiens (Human)
Instruction manual

FOR IN VITRO AND RESEARCH USE ONLY
NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

11th Edition (Revised in July, 2013)

[INTENDED USE]

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of bTG in human serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
TMB Substrate	1×9mL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Microplate reader with 450 ± 10nm filter.
2. Precision single or multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution

[STORAGE OF THE KITS]

1. **For unopened kit:** All the reagents should be kept according to the labels on vials. The **Standard**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20ºC upon receipt while the others should be at 4 ºC.
2. **For opened kit:** When the kit is opened, the remaining reagents still need to be stored according to the above storage condition. Besides, please return the unused wells to the foil pouch containing the desiccant pack, and reseal along entire edge of zip-seal.

Note:
It is highly recommended to use the remaining reagents within 1 month provided this is within the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable until this expiration date.

[SAMPLE COLLECTION AND STORAGE]

Serum - Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4ºC before centrifugation for 20 minutes at approximately 1000×g. Assay freshly prepared serum immediately or store samples in aliquot at -20ºC or -80ºC for later use. Avoid repeated freeze/thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2 - 8ºC within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20ºC or -80ºC for later use. Avoid repeated freeze/thaw cycles.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues were rinsed in ice-cold PBS(0.01mol/L,pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Minced the tissues to small pieces and homogenized them in 5-10mL of PBS with a glass homogenizer on ice(Micro Tissue Grinders woks, too). The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 5 minutes at 5000×g. Remove the supernate and assay immediately or aliquot and store at ≤-20ºC.

Cell Lysates - Cells must be lysed before assaying according to the following directions.

1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in cold PBS.
3. Resuspend cells in PBS (1×) and the cells was subject to ultrasonication for 4 times (or Freeze cells at ≤ -20ºC. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle for 3 times.)
4. Centrifuge at 1500×g for 10 minutes at 2 - 8ºC to remove cellular debris.

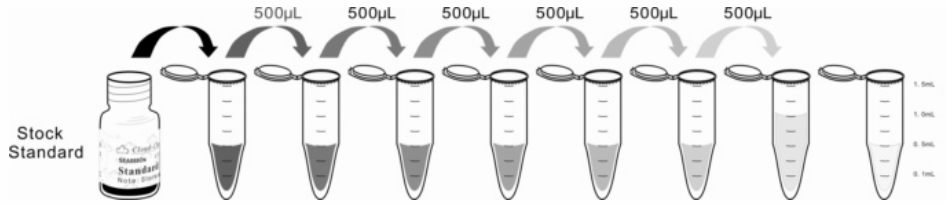
Cell culture supernates and other biological fluids - Centrifuge samples for 20 minutes at 1000×g. Remove particulates and assay immediately or store samples in aliquot at -20ºC or -80ºC for later use. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4ºC, otherwise samples must be stored at -20ºC (≤1 month) or -80ºC (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen should not be detected.
3. When performing the assay, bring samples to room temperature.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18-25ºC) before use.
2. **Standard -** Reconstitute the **Standard** with 1.0mL of **Standard Diluent,** kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 80,000pg/mL. Please firstly dilute the stock solution to 8,000pg/mL and the diluted standard serves as the highest standard (8,000pg/mL). Then prepare 7 tubes containing 0.5mL Standard Diluent and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 8,000pg/mL, 4,000pg/mL, 2,000pg/mL, 1,000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL, and the last EP tubes with **Standard Diluent** is the blank as 0pg/mL.



Tube	1	2	3	4	5	6	7	8	9
pg/mL	80,000	8,000	4,000	2,000	1,000	500	250	125	0

3. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with **Assay Diluent A** and **B,** respectively (1:100).
4. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
5. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standard within 15 minutes before assay. Please do not dissolve the reagents at 37°C directly.
3. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μL for once pipetting.
4. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
6. Contaminated water or container for reagent preparation will influence the detection result.

[SAMPLE PREPARATION]

1. Cloud-Clone Corp. is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. Serum/plasma samples require about a 50 fold dilution. A suggested 50-fold dilution is 10μL Sample + 490μL PBS. Sample should be diluted by 0.01mol/L PBS(PH=7.0-7.2).
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.

6. Due to the possibility of mismatching between antigen from other origin and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
7. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernatant may not be detected by the kit.
8. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, don't wash.
3. Add 100μL of **Detection Reagent A** working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.
4. Aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100μL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
6. Repeat the aspiration/wash process for total 5 times as conducted in step 4.
7. Add 90μL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
8. Add 50μL of **Stop Solution** to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20°C.
2. **Samples or reagents addition：Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.

3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes)**,** if the color is too deep, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Please protect it from light.
7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

[TEST PRINCIPLE]

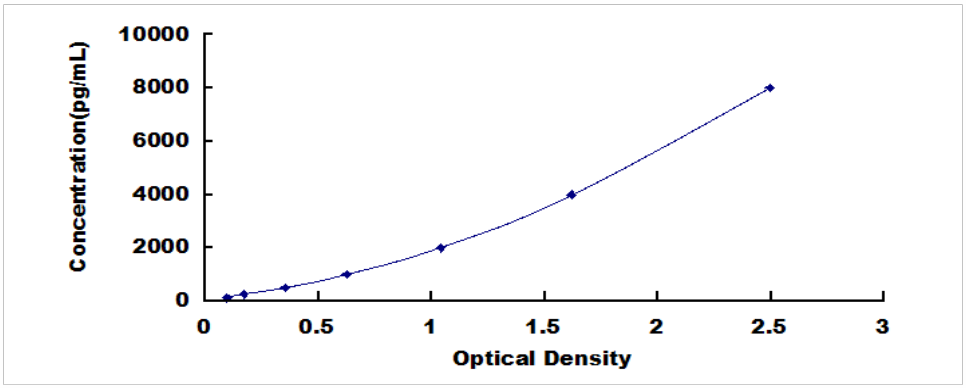
The microtiter plate provided in this kit has been pre-coated with an antibody specific to bTG. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to bTG. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain bTG, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of bTG in the samples is then determined by comparing the O.D. of the samples to the standard curve.

[CALCULATION OF RESULTS]

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D. and concentration for each standard and draw a best fit curve through the points on the graph or create a standard curve on log-log graph paper with bTG concentration on the y-axis and absorbance on the x-axis. Using some plot software, for instance, curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the known concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. However, the O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), plotting log of the data to establish standard curve for each test is recommended. Typical standard curve below is provided for reference only.



Typical Standard Curve for bTG, Human ELISA.

[DETECTION RANGE]

125-8,000pg/mL. The standard curve concentrations used for the ELISA's were 8,000pg/mL, 4,000pg/mL, 2,000pg/mL, 1,000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL.

[SENSITIVITY]

The minimum detectable dose of bTG is typically less than 53pg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

[SPECIFICITY]

This assay has high sensitivity and excellent specificity for detection of bTG.

No significant cross-reactivity or interference between bTG and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between bTG and all the analogues, therefore, cross reaction may still exist.

[RECOVERY]

Matrices listed below were spiked with certain level of recombinant bTG and the recovery rates were calculated by comparing the measured value to the expected amount of bTG in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=5)	78-92	85
EDTA plasma(n=5)	87-103	97
heparin plasma(n=5)	90-99	95

[LINEARITY]

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of bTG and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1：2	1：4	1：8	1：16
serum(n=5)	86-94%	82-101%	89-104%	80-94%
EDTA plasma(n=5)	91-105%	83-98%	80-94%	84-97%
heparin plasma(n=5)	84-97%	79-95%	96-105%	85-99%

[PRECISION]

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level bTG were tested 20 times on one plate, respectively.
Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level bTG were tested on 3 different plates, 8 replicates in each plate.
CV(%) = SD/meanX100
Intra-Assay: CV<10%
Inter-Assay: CV<12%

[STABILITY]

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage condition.
To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

[ASSAY PROCEDURE SUMMARY]

1. Prepare all reagents, samples and standards;
2. Add 100μL standard or sample to each well. Incubate 2 hours at 37°C;
3. Aspirate and add 100μL prepared Detection Reagent A. Incubate 1 hour at 37°C;
4. Aspirate and wash 3 times;
5. Add 100μL prepared Detection Reagent B. Incubate 30 minutes at 37°C;
6. Aspirate and wash 5 times;
7. Add 90μL Substrate Solution. Incubate 15-25 minutes at 37°C;
8. Add 50μL Stop Solution. Read at 450nm immediately.

[IMPORTANT NOTE]

1. Limited by the current condition and scientific technology, we can't completely conduct the comprehensive identification and analysis on the raw material provided by suppliers. So there might be some qualitative and technical risks to use the kit.
2. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
3. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for information.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.

5. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.
6. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
7. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. or greater at 450 ± 10nm wavelength is acceptable for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment. For more information, please refer to the operation video (http://www.cloud-clone.us/homepage/operate.htm).
8. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before assay for each batch is recommended.
9. Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
10. Kits from different manufacturers with the same item might produce different results, since we haven't compared our products with other manufacturers.
11. The instruction manual also suits for the kit of 48T, but all reagents of 48T kit are reduced by half.

[PRECAUTION]

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

[TROUBLE SHOOTING]

Problem	Possible Source	Correction Action
Poor Standard Curve	Improper standard curve preparation	Ensure accurate operation of the dilution
	Incomplete washing and aspiration	Adequate washing and adequate aspiration
	Inaccurate Pipetting	Check and Calibrate pipettes
Poor Precision	Incomplete washing of wells	Ensure sufficient washing
	Inadequate mixing and aspiration reagents	Adequate aspiration and mixing reagents
	Reused pipette tips, containers and sealers	Change and use new pipette tips, containers and sealers
	Inaccurate Pipetting	Check and Calibrate pipettes
Low O.D Values	Inadequate reagent volumes added to wells	Calibrate pipettes and Add adequate reagents
	Incorrect incubation times	Ensure sufficient incubation times
	Incorrect incubation temperature	Reagents balanced to room temperature
	Conjugate or substrate reagent failure	Mix conjugate &substrate,color should develop immediately
	No stop solution added	Follow the assay protocol in the kit manual
	Read beyond suggested reading time	Read within the time recommended in the manual
Sample Values	Improper Sample Storage	Store the sample properly and use the fresh sample
	Improper sample collection and preparation	Take proper sample collection and preparation method
	Low quantity of analyte in samples	Use new sample and repeat assay

Appendix-7: Manual of TXB2 ELISA assay

The assay protocol link:

http://www.abcam.com/ps/products/133/ab133022/documents/ab133022%20-%20Thromboxane%20B2%20ELISA%20Kit%20v7%20(website).pdf